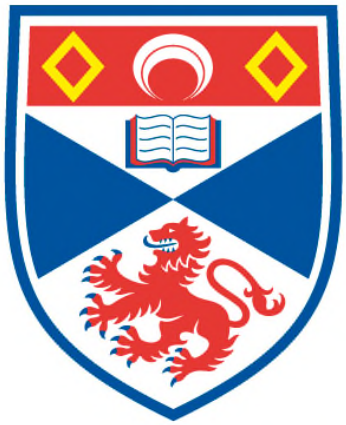
SEX DIFFERENCES IN ANXIETY DURING ADOLESCENCE: EVIDENCE FROM RODENTS AND HUMANS

Debra Alana Lynn

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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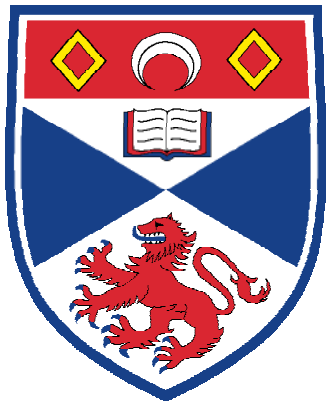
Sex differences in anxiety during adolescence: evidence from rodents and humans

By Debra Alana Lynn (030002771)

School of Psychology



University of St Andrews

A thesis submitted for the degree of PhD

on 17th February, 2012

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Abstract

Anxiety disorders commonly emerge during adolescence, and girls are diagnosed with these disorders more frequently than boys. Understanding why anxiety disorders emerge and why non-clinical anxiety symptoms increase during adolescence is important for understanding this sex difference and how to treat adolescent sufferers. Potential mechanisms, such as puberty or cognitive biases, can be investigated both in humans and in rodent models of anxiety. This thesis aimed to characterise sex differences and changes in anxiety-like behaviour across adolescence and into adulthood in the rat, and to examine anxiety and interpretive bias in adolescent humans. In rats, we examined performance on common tests of anxiety-like behaviour: the emergence test, open field and elevated plus-maze. Exploration on these tests increased from mid-adolescence into adulthood, and greater exploration by females was apparent from late adolescence. While the behaviours themselves remain interesting, caution on interpreting these behaviours as anxiety-related warranted and is discussed throughout the thesis. Potential effects of the ovarian cycle and testing order on EPM performance were also examined. In humans, 12-14 year old adolescents complete visual and written interpretive bias tasks, this bias being considered to be a cognitive vulnerability for anxiety. The results showed that, when imagining themselves in ambiguous scenarios, girls were more negative in their interpretations than boys. Additionally, both sexes also interpreted social scenarios more negatively than non-social scenarios. As puberty is thought to be important to the emergence of disorder during adolescence, interpretive bias could potentially mediate the puberty-anxiety relationship. While more interpretive bias work is needed in both species, the recent development of interpretive bias tasks for rodents provides an opportunity to move away from difficult to interpret tests of anxiety-like behaviour in rodents, and should allow for greater convergence of the human and rodent anxiety research.

Contents

Chapter 1: General Introduction

A. Anxiety in Humans

1. Anxiety

The Oxford English dictionary defines anxiety as “a feeling of worry, nervousness, or unease about something with an uncertain outcome”, for example, anxiety before completing an exam (O.E.D., 2010). For many people, anxiety is experienced as nervous butterflies in the stomach, sweating of the palms, a dry mouth, or even a restless night’s sleep before an important event like a job interview. While anxiety is a common emotion and both a natural and healthy response to many situations, in more extreme forms it can have detrimental effects on people’s lives.

Anxiety is considered to be a disorder when the anxiety experienced is prolonged or excessive in nature, and triggered by small events or stimuli that would not normally warrant an anxious response (American Psychiatric Association, 1994). A range of clinical anxiety disorders exist, varying according to how that anxiety manifests itself emotionally and behaviourally, and in terms of the different stimuli or experiences that induce anxiety. For example, some people experience anxiety in terms of extreme physical feelings when they are stressed, including heart palpitations, the sensation of choking and shortness of breath, as seen in panic attacks. For others, a specific object or situation, such as spiders or interacting with new people, produces irrational anxiety (known as a phobia) to such a degree that the phobia affects that person’s daily life and their behaviour when they are, or even anticipate, being exposed to that stressor (American Psychiatric Association, 1994). As will be discussed, these disorders are statistically common – in fact, nearly one in four of us is likely to experience clinical levels of anxiety in our lifetime (Bremner, 2004).

When looking at men and women, research has shown that women are almost twice as likely to experience an anxiety disorder during their lifetime as are men (Kessler et al., 2005). In addition, many of the anxiety disorders first appear during adolescence (e.g. Kessler et al., 2005), as does the increased frequency of anxiety amongst females as compared to males (e.g. Breton et al.,

1999). Adolescence is the gradual period of transition that bridges childhood and adulthood, during which the individual develops and learns new skills in order to become independent of their parents. By and large, this important developmental period occurs approximately between the ages of 12-18 years old (Spear, 2000). This thesis focuses upon why adolescence is such an important developmental period in the emergence of many anxiety disorders, considering why some of the physiological changes that occur during adolescence may be so important.

With this in mind, the following section reviews the frequency of anxiety disorders in the general population (known as the prevalence rate), both with regards to adolescence and to how this period of life compares to childhood and adulthood. The potential ways that adolescence may be so important for the emergence of anxiety disorders are reviewed, with special attention paid to the role of hormonal, neural and cognitive changes occurring at this time.

2. Prevalence of Anxiety Disorders

The diagnosis of anxiety disorders has been standardised by the wide use of the Diagnostic and Statistical Manual of Mental Disorders (fourth edition - the DSM-IV; American Psychiatric Association, 1994). This manual is used both in North American health systems and in research internationally. The DSM-IV combines disorders under the umbrella term of anxiety on the basis that the anxiety experienced is prolonged or excessive in its nature, and that the anxiety therefore interferes significantly with the person's normal routine, occupational or academic functioning, social activities or any other import areas of functioning. Furthermore, the anxiety must not be the result of any other underlying medical illnesses or medications. While a list of specific sub-disorders are diagnostically defined and outlined by DSM-IV (such as specific phobia, post-traumatic stress disorder and so on), most of the work reviewed below will consider the prevalence of the spectrum of anxiety disorders as a whole, focusing only on some specific disorder types where appropriate.

Anxiety disorders are the most commonly diagnosed spectrum of disorders as outlined by the DSM-IV. For example, a large scale study in the

U.S. (known as the National Comorbidity Survey Replication) involving 9282 participants aged 18 and over found anxiety disorders to have a lifetime prevalence rate of 28.8% (Kessler et al., 2005). This is closely followed by mood disorders (which include depression and bipolar disorder) with a 20.8% prevalence rate, but is much greater than other disorder families, such as substance use disorders (14.6%). In addition to this, Kessler and colleagues analysed sociodemographic predictors of the lifetime prevalence of anxiety disorders, finding that women were significantly more likely to experience clinical anxiety during their lifetime relative to men in a ratio of up to 1.8 (women) to 1 (men). While prevalence studies are undeniably affected by biases in the characteristics of those who choose to take part and in terms of what questions will be answered honestly given the potential for embarrassment, Kessler and colleagues have carefully addressed these issues. The 29.1% of invitees who chose not to take part were sent a non-response questionnaire to determine the characteristics of this population. Contrary to the belief that the mentally ill avoid taking part in such research, no evidence of this bias was found. In addition to this, several measures were taken to reduce the embarrassment of answering difficult questions and to increase the accuracy of participants' responses, so addressing the avoidance and misunderstanding of embarrassing or difficult questions. Along with the large sample size, this study is therefore of great utility and merit in this field.

While Kessler and colleagues' (2005) publication may be the seminal paper of recent times on the prevalence of psychiatric disorders, this study is not the only paper to have found such results. Somers, Goldner, Waraich and Hsu (2006) performed a systematic review of 41 anxiety prevalence studies conducted between 1980 and 2004 in a variety of countries. Amongst the qualifying criteria for inclusion were that the sample size had to be at least 450, and either current diagnostic criteria or clinician diagnoses were required. From each qualifying publication, information on overall, age-specific and sex-specific prevalence rates were then extracted and analysed. Somers and colleagues (2006) reported that the lifetime prevalence rate for anxiety disorders was 16.6% (95% confidence intervals of 12.7-21.1 %), which is somewhat lower

than Kessler and colleagues' estimate of 28.8%. In terms of one year prevalence rates for all anxiety disorders, women were also found to suffer more frequently than men in a ratio of approximately 2 to 1, consistent with Kessler and colleagues' findings.

Anxiety disorders are not solely experienced in adulthood however, and are also prevalent among adolescents. Costello, Mustillo, Erkanli, Keeler and Angold (2003) conducted a study of 1420 children aged 9-12 years old, assessing their mental health until 16 years of age as part of the Great Smoky Mountains Study in the U.S. These researchers calculated the cumulative prevalence of DSM-IV disorders by the age of sixteen and found that, by this age, 9.9 % of the adolescents had met the criteria for an anxiety disorder. Also, in Kessler and colleagues' National Comorbidity Replication Study (2005), the age-of-onset distributions revealed that 75% of individuals with anxiety disorders are diagnosed before they reach the age of 21.

Similarly to adulthood, sex differences in the prevalence of anxiety disorders exist in adolescents. In Costello and colleagues' (2003) study, while the overall prevalence rate for anxiety disorders was 9.9% by the age of 16, when this rate was calculated separately for each sex, 12.1% of girls met the criteria for clinical anxiety compared to only 7.7 % for boys. The same is not true of childhood however, with research generally indicating that there are no or few sex differences in anxiety during childhood, but that the sex differences emerge during the adolescent period (Paus, Keshavan & Giedd, 2008). A large scale study of mental disorders in 6-14 year old children conducted in Quebec, Canada (Breton et al., 1999) revealed that in terms of sex differences, while boys and girls did not differ in terms of the rate of anxiety disorders in childhood (6-10 years), girls did have a higher rate of anxiety disorders than boys in the 9-14 year old groups. These results suggest that sex differences emerge during the adolescent period.

Focusing on adolescence in particular, this period not only sees the emergence of anxiety disorders in general, but certain specific subtypes are particularly common during this life period, especially social anxiety disorder. The DSM-IV defines social anxiety disorder (also known as social phobia) as

the persistent fear of one or more social or performance situations where there is exposure to unfamiliar people or to potential scrutiny. Exposure to such situations induces anxiety, so the sufferer is either avoidant of such situations or endures them under intense distress and anxiety (American Psychiatric Association, 1994). Kessler and colleagues (2005) report that 75% of the individuals who suffer from social phobia meet the criteria for the disorder by 15 years of age.

On the other side of the coin, not only is social phobia frequently diagnosed in adolescence, but new cases of the disorder are rarely diagnosed in adulthood. For example, Neufeld, Swartz, Bienvenu, Eaton and Cai (1999) interviewed 1920 subjects who had taken part in the first wave of a psychiatric survey in 1981. The authors examined the incidence of social phobia (incidence being the number of new cases being seen, compared to prevalence which is typically the percentage of people who already have a diagnosis within a certain period of time), and found that the incidence rate among adult was only 4-5 per 1000 people per year. Neufeld and colleagues' results therefore fit with Kessler and colleague's findings that 75% of cases of social phobia are seen by the age of 15, and in fact by early adulthood (23 years of age) 90% of the cases of social anxiety disorder are already diagnosed.

In addition to social phobia being common during adolescence, there is also a sex difference in the frequency of its diagnosis, with girls being more likely to suffer from the disorder than are boys. A large scale longitudinal study conducted in Germany examined social phobia in 3021 participants aged 14-24 years old over a 30 month period. Both the 12 month prevalence rates (7.2% in females, 3.2 % in males) and lifetime cumulative incidence rates (9.5% in females, 4.9% males) were significantly greater in females compared to males (Wittchen, Nelson & Lachner, 1998). Magee, Eaton, Wittchen, McGonagle and Kessler (1996) have also shown lifetime prevalence rates of social phobia to be higher in adolescent and young adult participants compared to older cohorts.

While the research reviewed above indicates that social phobia is a common anxiety disorder and that it often first emerges during adolescence, this is not the case for all of the anxiety disorder subtypes. For example,

separation anxiety (whereby the sufferer experiences unreasonable anxiety in the face of leaving their home or leaving someone to whom they are attached) is essentially a disorder of childhood. For example, Kessler and colleagues (2005) showed that 75% of individuals with separation anxiety disorder meet the criteria by the age of 10 years old. There is also evidence that the prevalence of this disorder decreases around the period of adolescence. For example, Breton and colleagues (1999) found that for 6-8 year olds there was a six month prevalence rate of 4.9% for separation anxiety disorder, decreasing with age and reaching a nadir of 1.3% in 12-14 year olds (Breton et al., 1999). Generalised anxiety disorder (referred to by Breton et al. as overanxious disorder, according to its previous title in the DSM-III) also followed a similar pattern, with children having higher prevalence rates of this disorder (3.8 - 3.9 per 100 in 6-11 year olds) than did early adolescents (1.7 per 100 in 12-14 year olds).

In summary, adolescence is obviously not the only period of life where anxiety disorders emerge. Children also experience anxiety disorders (separation anxiety specifically so), and for many of the specific subtypes the DSM-IV clearly indicates how children's symptoms may differ from adults but are still regarded as diagnostic. While it is beyond the scope of this thesis, adulthood is also an undeniably important time of life with regards to the diagnosis of anxiety disorders. Nonetheless, adolescence remains an important period of life in the study of clinical anxiety, and perhaps social phobia especially so.

3. Potential Mechanisms Underlying the Emergence of Anxiety during Adolescence

The period of adolescence is associated with a suite of physical, neural and behavioural changes. Puberty, the process of sexual maturation, is a very important endocrine event that occurs during adolescence, as are the associated changes in the structure and activity of the brain, as well as shifts in cognition. The potential importance of these changes during adolescence in terms of making adolescents vulnerable to experiencing anxiety disorders will

be reviewed below, including a brief discussion of areas of the brain which are associated with anxiety.

3.1 The Role of Puberty

The gonadal hormones estrogen and progesterone have long been suggested to play an important role in why women are more likely to experience anxiety than men. For instance, with regards to the menstrual cycle, positive affective state has been reported to increase around the time of ovulation (Sanders et al., 1983), while levels of anxiety can increase during the premenstrual phase (Ivey & Bardwick, 1968). Anxiety disorders have also been found to increase in other times of hormonal flux in women's lives, including post-partum (e.g. Wenzel, Haugen, Jackson & Brendle, 2005) and at menopause (e.g. Cagnacci et al., 1997; Freeman et al., 2005). Therefore, it is plausible that the process of puberty, whereby the body goes through a suite of endocrine changes in order to achieve sexual maturity, may have an important role to play in the emergence of anxiety disorders during adolescence.

Puberty is a complex physiological process that begins in early adolescence when the hypothalamus begins to release increasing amounts of gonadotropin-releasing hormone (GnRH). These pulses of GnRH release are responsible for stimulating the pituitary gland to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH in turn stimulate the gonads to release the steroid hormones testosterone (from the testes in males) or estrogen and progesterone (from the ovaries in females; Ojeda, Andrews, Advis & White, 1980). As a result of these increases in circulating hormones, many important morphological changes occur. Both boys and girls reach skeletal maturity by going through a growth spurt (Rogol, Roemmich & Clark, 2002), and both sexes experience the growth of body hair (including facial hair in boys), pubic hair and undergo changes in the skin. For girls, menarche (first menstruation) is a key event, and the breasts also develop into their adult form at this time. For boys, genital and testicular growth occurs, along with the first emission of sperm (spermarche), first ejaculation (known as oigarche) and deepening of the voice (Johnson, 2007).

In terms of the research linking puberty and anxiety, two main approaches have been taken. The first has been to examine the relationship between anxiety and the current degree of progression through puberty as indexed by the morphological changes - a measure known as pubertal status. This research on the relationship between pubertal status and anxiety is reviewed below. The second approach involves assessing the effect of the age of pubertal onset on anxiety; this is referred to as pubertal timing (for example, examining whether individuals that begin puberty relatively early are more likely to develop clinical anxiety than on-time or late developers). As pubertal timing is not relevant to this thesis, the literature on pubertal timing and anxiety will not be discussed further.

3.1.1 Pubertal Status and Anxiety

As adolescence is an important period in the emergence of clinical anxiety disorders, it follows that there should be shifts in anxiety in the non-clinical adolescent population (of which some will obviously develop into clinical cases). This section considers the literature on the relationship between pubertal status and anxiety symptoms in these non-clinical, typically developing adolescents.

Reardon, Leen-Feldner and Hayward (2009) have conducted an extensive review of the anxiety and pubertal status literature, and concluded that, in general for girls, the likelihood of anxiety symptoms being reported increases with pubertal status. In other words, girls who are further progressed through puberty report being more anxious. While there is much less consistent evidence for boys, Reardon and colleagues (2009) suggest that the trend appears to be the same. However, not all studies were consistent in their findings. One possible explanation for the disparity in results could be the use of different types of anxiety-related measures. Those studies that used clinical diagnostic measures, such as interviews, to measure anxiety symptomatology generally reported that anxiety increases with pubertal status. For example, Hayward and colleagues (1992) examined anxiety and pubertal status in 754 girls aged 10-15 years using a diagnostic measure (the Structured Clinical Interview for DSM-IV Disorders) and found a significant relationship between

panic attack history and pubertal status, where the risk of having suffered a panic attack doubled for every one stage increase in pubertal status. Similarly, Ge, Brody, Conger and Simons (2006) reported that increasing pubertal status was positively correlated with generalised anxiety symptoms in both boys and girls (10-12 years old; 400 boys, 467 girls) using the Diagnostic Interview Schedule. Importantly, both of these studies also controlled for chronological age, indicating that above and beyond any age differences, more developed adolescents seem to be more anxious.

In contrast, those studies using tests that are not designed to clinically diagnose anxiety disorders but merely to measure anxiety symptoms, appear to give much more mixed results. Of four relevant studies using non-clinical anxiety measures, two have found a positive relationship between pubertal status and anxiety symptoms (Ge, Conger & Elder, 2001; Patton et al., 1996) and two have found either the opposite or no relationship (McCabe, Ricciardelli & Banfield, 2001; Stone & Barker, 1939). For instance, McCabe and colleagues (2001) found no link between pubertal status and anxiety for both boys and girls despite a large sample size (N = 1185). This may be due to the choice of measure (the anxiety portion of the Depression Anxiety Stress Scales) which does not bear a direct relationship to diagnostic categories of anxiety. Also, despite using a wider range of ages (12-16 years old), age was not controlled for in this study, which is essential given the potential difference between age-related and development-related changes.

In summary, the research seems to show that in general girls (and to a lesser extent, boys) are more likely to suffer anxiety symptoms the further through puberty they have progressed. Boys may potentially be less affected by the process of puberty in terms of their vulnerability to anxiety compared to girls. Boys may also respond differently to self-reporting their pubertal status and anxiety symptoms however. Noticeably the study that showed a positive relationship between anxiety and pubertal progression in boys (Ge et al., 2001) was longitudinal in nature, where ‘practice’ effects of repeating the same measures annually may have altered how the boys responded. Methodologically, to help detect differences in anxiety across puberty a

diagnostic anxiety measure may be required (compared to non-diagnostic and general psychiatric symptoms measures), preferably aided by a large sample size. Chronological age also needs to be considered in such studies, either by controlling for age statistically or by using limited age ranges for comparison. In future, this field of research also needs to consider concomitant changes at this time which may interact with puberty to have effects on anxiety, given that puberty is not pathological; while every healthy child experiences puberty, not every child develops an anxiety disorder (Leen-Feldner, Reardon, Hayward & Smith, 2008).

3.2 The Role of Neural Development

During adolescence, the human brain undergoes substantial development and reorganisation (Blakemore & Choudhury, 2006; Toga, Thompson & Sowell, 2006). Some of these developmental changes are dimorphic between the sexes; for instance, the volume of grey matter peaks earlier in boys than girls (Giedd et al., 1999; Lenroot & Giedd, 2010), and recent evidence suggests that gonadal hormones released during puberty could play a role in the re-organisation of the brain during this period of life (e.g. Bramen et al., 2011; Neufang et al., 2009; Peper et al., 2009; reviewed by Blakemore, Burnett & Dahl, 2010). Therefore, the increased prevalence of anxiety disorders during adolescence may be related to the fact that there are significant developmental changes occurring in brain regions that are known to be involved in anxiety.

Neuroimaging studies involving a wide array of clinically anxious patient groups converge to indicate the role of two main areas of the brain: the amygdala and the prefrontal cortex (Bishop, 2007; Mathew, Price & Charney, 2008). The amygdala is a limbic region primarily involved in co-ordinating responses to threat. In terms of the brain's circuitry, the amygdala is reciprocally connected to prefrontal areas, with amygdala activity appearing to be influenced in a top-down manner by inputs from the prefrontal cortex, as well as by inputs from the hippocampus (a structure largely involved in memory; Rauch, Shin & Wright, 2003). During adolescence, the amygdala changes in volume and becomes significantly larger in boys than girls, after accounting for total brain

volume differences between the sexes (Neufang et al., 2009; Peper et al., 2009).

The prefrontal cortex (PFC) is an area of the brain that is involved in many functions, including attention control, language, memory and behavioural inhibition (Fuster, 2001). The PFC is considered to be largely involved in anxiety in terms of biasing attention towards threatening information, and these attentional biases are known to be commonplace amongst those suffering from anxiety (Blanchette & Richards, 2010; Mathews & MacLeod, 2005). While there is still only limited evidence for anatomical changes in the PFC during adolescence, such research does suggest that PFC development differs between the sexes (Raznahan et al., 2010). This brain region also appears to function differently in boys compared to girls, as sex differences have been found in tasks that heavily rely on the PFC (Overman, 2004).

Gonadal hormones may play an important role in the development of sex differences in brain structures and functions. Testosterone, progesterone and estrogen are capable of crossing the blood-brain barrier and can affect behaviour by altering gene expression in neural cells or by directing neurotransmission and other neural processes (McEwen, 1981). These steroid hormones can play a role in organising brain structures by directing processes such as neuronal survival, neurogenesis and synaptogenesis, producing long-term effects on neural circuits and subsequent behaviour (MacLusky & Naftolin, 1981; Wright, Schwarz, Dean & McCarthy, 2010). One way that steroid hormones achieve such effects is via activation of specific estrogen, progesterone or androgen receptors. For example, the amygdala is known to have a particularly high concentration of androgen receptors (Sarkey et al., 2008), which may have a role to play in sex differences in anxiety.

In addition to these direct effects of gonadal hormones on the brain which may be relevant to anxiety, these same hormones may also have more indirect effects. For example, gonadal hormones may be involved in changes in sleeping pattern (Manber & Armitage, 1999) and body weight (Rosenbaum & Leibel, 1999), and these in turn may have an affect on anxiety itself or at least susceptibility to experiencing anxiety.

In summary, adolescence may be an important period in the development of anxiety due to changes in the brain. Many of the neural changes have been found to be sexually dimorphic in nature, and gonadal hormones are capable of having effects on these relevant brain structures. This research provides further support that puberty constitutes an important event in the increase in anxiety disorder prevalence during adolescence.

3.3 Cognitive Factors: Interpretive Bias

As reviewed above, the brain undergoes a variety of anxiety-related neural changes during adolescence. Following on from this, it may then be the case that cognitive processes sub-served by these same areas may also change during this period of life. An important and robustly reported cognitive process that is associated with anxiety is interpretive bias, whereby ambiguous information is assumed to be threatening in nature (Blanchette & Richards, 2010; Mathews & MacLeod, 2005). For example, a noise downstairs is assumed to be an intruder rather than the pet cat, or an email request to meet with the boss is taken as an indication of being in trouble, rather than merely meeting to discuss a new project. Much more work on the neural circuits underlying this bias is needed, but some research already suggests that the interplay between the amygdala and the prefrontal cortex may be the mechanism by which interpretive biases occur (Bishop, 2007).

Interpretive bias has been found in a variety of anxious participant groups, using a range of methodologies. For instance, Mathews, Richards and Eysenck (1989) presented generalized anxiety disorder (GAD) patients, recovered anxious participants and non-anxious controls with threat-neutral homophones in audio format (such as die/dye, bury/berry, guilt/gilt). The number of threat interpretations made by participants was used as a measure of interpretive bias, with the GAD patients endorsing significantly more threatening meanings than the non-anxious controls (Mathews et al., 1989). The scores of the recovered participants were intermediate and not significantly different from either of the other two groups. The same has been found in other studies using similar methodologies (e.g. Richards & French, 1992).

Another approach to measuring interpretive bias has been to present participants with ambiguous scenarios and ask the participant to imagine him- or herself in that situation. For example, one scenario might be 'you are yet to receive any replies to invitations for your party next weekend'. The participant can then either be asked the reasons why this might be so (including which of these reasons he/she believes is the truth) or to rate possible explanations already provided by the experimenters. These responses can be positive ('they all want to come – they don't need to tell me that'), neutral ('it's too early to hear back yet') or negative in nature ('no one wants to come to my party').

Social anxiety and its corresponding disorder have been particularly focused upon using this ambiguous scenario design. This is most likely due to the frequent ambiguities which occur in social interaction that can lead to negative interpretations (such as a loved one not returning a call, or a friend turning down an offer to meet up) and often result in the avoidance of social situations ('I won't go to the party because no one will want to talk to me'). Although less information is available using participants with social anxiety disorder, work with participants reporting high and low levels of social anxiety has revealed robust interpretive biases. For example, Brendle and Wenzel (2004) assessed 54 self-reported socially anxious participants and 58 who were not highly socially anxious. Assessing the valence of participants' responses to brief scenarios, the socially anxious group were both less positive and more negative in response to all the passages that they were presented with. Similar results have also been found by other researchers (e.g.; Vassilopoulos, 2006).

The ambiguous scenario method has also been used to examine content-specificity for social anxiety (in other words, whether socially anxious individuals make negative interpretations only for social situations). Constans, Penn, Ihen and Hope (1999) found that high socially anxious participants interpreted social scenarios more negatively than a low-socially anxious group, but that no differences were found for non-social scenarios (N=94). This content-specificity for biased social interpretations in social anxiety has also been reported in other studies (e.g. Huppert, Foa, Furr, Filip & Mathews, 2003).

More importantly for this thesis however, interpretive bias has also been reported in adolescent participants. For example, Creswell, Schniering and Rapee (2005) compared the performance of clinically anxious participants with a range of anxiety diagnoses (N = 60, mean participant age of 11 years) to a non-anxious controls on an ambiguous scenario task. This study found that self-rated anxiety across all participants correlated positively with threat interpretation (in other words, the more anxious the participant, the more negative threat interpretations they made). Similarly, Bögels and Zigterman (2000) used a mixed clinical anxiety group of adolescents, as well as non-anxious and diagnosis controls (adolescents with behavioural disorders). The results revealed that, relative to both control groups, the anxious participants underestimated their ability to cope in the scenarios they imagined themselves experiencing, and rated the scenarios higher for danger.

Like the adult interpretive bias literature, the adolescent research has commonly focused upon social anxiety and social phobia (a disorder that is particularly prevalent during this period of life, as already discussed). Similarly to adults, socially anxious adolescents have been found to exhibit interpretive biases when confronted with social scenarios. For instance, Rheingold, Herbert and Franklin (2003) assessed interpretive bias in 12-17 year old participants with a social anxiety disorder diagnosis (N = 37) and in same-aged non-anxious controls (N = 22). The results showed that the socially anxious participants both over-estimated the cost of negative social events and the likelihood of the imagined negative event happening to them compared to the non-anxious controls. Similarly, in a study of 11-16 year olds, Miers, Blöte, Bögels and Westenberg (2008) reported that adolescents scoring high on social anxiety (N = 37) were more negative in their interpretations of ambiguous social scenarios compared to a group of control participants (N = 36) that had average social anxiety scores.

While all the interpretive bias studies involving adolescents reviewed thus far included both sexes, most did not investigate whether there were any sex differences. Miers and colleagues (2008), however, reported that adolescent girls were both less positive and more negative in their interpretation

of social scenarios than same-aged boys, while no sex difference was found for non-social scenarios. Therefore, interpretive bias can be seen in socially anxious adolescents as well as adults, in both clinical and non-clinical participant groups. While the evidence for content-specificity is more mixed in adolescents than in adults (Muris & Field, 2008), this may be a product of different interpretive bias measures and sample sizes, as well as social anxiety being less well crystallised as a disorder in adolescence or due to the selection of adolescents with sub-clinical symptoms in contrast to older sufferers.

One of the issues that remains is whether interpretive bias is something that arises or changes during adolescence, a time when many anxiety disorders emerge. No research to our knowledge has addressed the emergence or alteration of interpretive bias across development, and none has considered the potential importance of puberty. Given that puberty is a normative process and is not pathological for anxiety in itself, interpretive bias may represent an important variable that could mediate the relationship between anxiety and puberty. Sex differences during adolescence also need further consideration, given that while both sexes are included in much of the research, rarely is sex analysed or reported as a statistical factor.

4. Summary

In summary, anxiety disorders are of interest because of their high prevalence, particularly in adolescents. There are several potential reasons as to why this period of life is so important, including puberty (both its somatic and neural consequences) as well as a range of cognitive changes, all of which are likely inter-related. Focusing on the role of puberty however, lots of questions remain as to its importance in the emergence of anxiety and sex differences in adolescents. In addition, the role that other factors may have to play in mediating the puberty-anxiety relationship are in need of further study, given that puberty is a normative process rather than a risk-factor for anxiety. The next part of this thesis considers the utility of using rodent models to further investigate the importance of adolescence and puberty to anxiety, and evaluates how anxiety is measured in non-human animals. The current

evidence for changes in anxiety-like behaviour across adolescence and the emergence and existence of sex differences in such behaviours are also reviewed.

B. Anxiety-like Behaviour in Rodents

There are a variety of ways to approach the research topic of anxiety changes during adolescence in humans, which include using a rat as a potential model of behavioural development across adolescence. Rats are appropriate models for two reasons: firstly, rats go through adolescence and puberty, as do humans and pubertal development is governed by the same gonadal hormones. Rats can therefore provide methodological flexibility in terms of hormonal measurement and manipulation. Secondly, rats are already widely used in preclinical research to develop new pharmacological drugs that can treat anxiety in humans. Not only is this a direct benefit from the use of rats as a model of anxiety, but it also means that there is a wide literature upon which further anxiety-related research can draw, including a range of widely-used behavioural tests. This pharmacological research still needs to work towards developing adolescent-appropriate anxiolytic medications however; this is important given that significant negative side-effects have been reported for adolescents using anxiolytic drugs that have been tested on and developed for adults (Hammad, Laughren & Racoosin, 2006; Malkesman et al., 2009). Knowing how behaviours relevant to anxiety in the rat develop across the adolescent period is therefore a prerequisite to examining the effects that certain anxiety-affecting compounds have on rats during this stage of life.

5. Adolescence and Puberty in the Rat

Similarly to humans, adolescence is the developmental period spanning between the juvenile and adult phases of a rat's life, where independence from the dam is obtained (Sisk & Zehr, 2005). During this time, the process of sexual maturation takes place whereby a suite of physical and physiological changes occur, including changes in body size, neural anatomy and hormone levels.

Alongside these differences, salient behavioural changes are also occurring, which include play, exploration away from the home burrow, and interaction with unfamiliar conspecifics (Calhoun, 1963).

In physical terms, adolescence is considered to begin around the time of weaning, which occurs on post-natal day (pnd) 21 in laboratory rats (Tirelli, Laviola & Adriani, 2003). At this time until approximately pnd 34, gonadal hormone levels (estrogen, progesterone and testosterone) are rising in both male and female rats, and the gonads are increasing in weight (Gabriel, Roncancio, & Ruiz, 1992). Behaviourally at this time, young rats become reliant on solid food rather than maternal milk (Galef, 1981) and, in the wild, will explore the area immediately outside of the natal burrow system (Calhoun 1963). Play behaviour (a simulation of attack and defence) is also frequently exhibited in rats of this age, and continues to form much of an adolescent rat's behaviour until pnd 40 (Pellis, Pellis & Whishaw, 1992).

During mid-adolescence (pnd 34-46), female rats exhibit irregular ovarian cycles and experience vaginal patency (the opening of the vagina) while male rats continue to experience increases in testosterone (Gabriel et al., 1992). During this period, both sexes travel further from the home burrow system, joining their dam on foraging trips (Calhoun, 1963). Late adolescence spans pnd 46-60. During this time, female rats begin to show regular ovarian cycling and male rates produce fertile sperm (Gabriel et al., 1992; Tentler et al., 1997). At pnd 60, young rats are considered to be sexually mature (Zemunik et al., 2003), and will engage in sexual and aggressive interactions with other adult rats (Calhoun, 1963). Testicular development does continue past pnd 60 however, with peak testosterone levels being reported at pnd 70 before reducing slightly to typical adult levels (Knorr, Vanha-Perttula & Lipsett, 1970). Late adolescents also explore away from their burrow without the company of their dam, and interact with novel conspecifics (Calhoun, 1963).

5.1 Neural Development During Adolescence in the Rat

As in human beings, the rodent brain undergoes substantial development and reorganisation during adolescence (Brenhouse & Andersen, 2011; McCutcheon

& Marinelli, 2009; Sisk & Zehr, 2005). Areas of the rodent brain that change during the adolescent period include those areas that have been associated with anxiety in human beings - the amygdala and prefrontal cortex. For example, the medial amygdala increases in volume from adolescence to adulthood in Syrian hamsters (Romeo & Sisk, 2001), and, in rats, dopamine receptors in the prefrontal cortex are initially over-produced, and then pruned, during the adolescent period (Andersen et al., 2000). Some of these changes are dimorphic between the sexes; for instance, the adolescent overproduction and pruning of dopamine receptors is more pronounced in male rats than in same-aged females (Andersen, Rutstein, Benzo, Hostetter & Teicher, 1997).

Gonadal hormones have been proposed to play a major role in organising the rodent brain during adolescence, particularly sexually dimorphic structures (Schulz, Molenda-Figueira & Sisk, 2009), and recent experimental evidence has supported this hypothesis (e.g. Ahmed et al., 2008; Cooke, 2011; Romeo, Diedrich & Sisk, 2000; Yates & Juraska, 2008). For example, rising levels of testosterone during adolescence have been shown to enhance neuronal number and synaptic density in the medial amygdala of male rats and hamsters (Ahmed et al., 2008; Cooke, 2011). The involvement of gonadal hormones in the development of sex differences in the brain does not exclude other factors, such as genes and experiential factors, from also being involved (McCarthy & Arnold, 2011).

6. Measuring Anxiety-like Behaviour in the Rat

Anxiety in humans can be defined as an over-estimation or bias towards personal threat that leads to apprehension and a nervous emotional reaction (Stephan, Stephan & Gudykunst, 1999). As anxiety in humans necessarily involves conscious feelings (i.e. “I feel anxious”), it is not know whether rats experience anxiety in the same way. However, removing this component of subjective feeling, rats do share the physiological, cognitive and behavioural components of emotional responses with humans (Mendl & Paul, 2004). As a predated species, rats both evaluate and respond to potential and real threats in their environment. In behavioural terms, anxiety-relevant behaviour in the rat

has been defined as behaviour that permits approach to potential threat, such as entering an open area where the risk of predation is higher (McNaughton & Zangrossi, 2008). In contrast, fear is where animals exhibit avoidance and flight in response to real danger, such as fleeing upon detecting the scent of a predator (McNaughton & Zangrossi, 2008). This thesis thus refers to 'anxiety-like' behaviour when discussing the measurement of anxiety in rats, due to this necessary difference in how anxiety is defined in humans compared to the rat. Where the term ‘exploration’ is used, this denotes the entering of different areas of an apparatus, in other words, it is used as a synonym of locomotion, and not as a term involving motivation.

A range of unconditioned behavioural tests have been used to examine anxiety-like responses in the rat, many of which are frequently employed by behavioural pharmacologists in the development of anxiolytic drugs. All of these tests work on the basis that the rat is a predated species, and as such avoids open, exposed, and well lit areas when possible, as it may be more visible to potential predators. Three common tests of anxiety-like behaviour will be reviewed below: the open field, the emergence test and the elevated plus-maze. The apparatus will be described, the literature on sex and age difference in performance will be briefly reviewed, and the strengths and weaknesses of these behavioural measures of anxiety-like behaviour will be discussed.

6.1 The Open Field (OF)

The OF is one of the oldest behavioural tests of anxiety-like behaviour. Developed from Hall’s original test (1934), the open field consists of a novel walled space in which the rodent is placed for a set period of time by an experimenter and cannot escape (hence the OF being referred to as a ‘forced’ test; Welker, 1957). The apparatus is divided into sections by physically drawn or camera-imposed lines that map out the exposed, central square and the more protected perimeter immediately next to the wall (**Figure 1.1**).

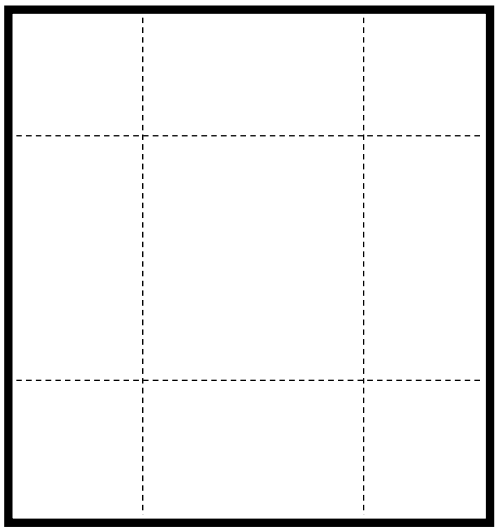

Figure 1.1 Line diagram of the open field, with dashed lines representing the sub-sections of the OF as used in our laboratory.

In the wild, rats naturally prefer to take covered paths that are bordered by objects that provide some degree of shelter, and avoid venturing into open spaces as the predation risk there is higher (Calhoun, 1963). The behaviours of interest measured by the open field are the number of entries into the centre square of the arena, as well as how long a rodent is prepared to spend in this more exposed central region. An animal that spends the majority of its time in the regions next to the walls and rarely enters or spends much time in the centre is assumed by some researchers to be exhibiting a high anxiety-like behaviour, whereas an animal making more visits to the centre of the apparatus and spending more time in the centre relative to other animals is described as exhibiting low anxiety-like behaviour. In support of activity in the centre of the OF being relevant to anxiety, drugs known to decrease anxiety in human beings, such as benzodiazepines and seratonergic-effecting medications, have often been shown to increase the number of entries into or proportion of time spent in the centre of the OF (Prut & Belzung, 2003).

Studies of adult rats have generally found that adult females make more entries into the centre of the OF and spend more time there compared to males (e.g. Beatty, 1979). In addition, females are also found to generally locomote

more than males in the open field (e.g. Blizard, Lippman & Chen, 1975; Slob, Huizer & Van der Werff Ten Bosch, 1986). Research on the behaviour of adolescents in the open field has produced inconsistent results about the level of anxiety-like behaviour relative to adult rats. Adolescents have been reported to exhibit higher levels (Arakawa, 2005; Bronstein, 1972; Philpot & Wecker, 2008) or lower levels (Candland & Campbell, 1962) of centre square entries and duration in the OF compared to adults. With regards to sex differences in these behaviours potentially emerging during adolescence, even less is known about how male and female adolescents behave on this test. Some studies have reported that adolescent females behave similarly to same-age males (Candland & Campbell 1962; Masur, Schutz. & Boerngen, 1980; Renner et al. 1992; Slob et al. 1986). One study has however reported that adolescent female rats were generally more active in the OF than male subjects of the same age, although activity in the centre of the apparatus was not reported (Kierniesky, Sick & Kruppenbacher, 1977). Further research is therefore warranted to examine the differences between adult and adolescent behaviour on the OF, and to examine any potential sex differences across puberty and adolescence.

One of the main advantages of the open field is its ease of use. Not only is the OF cheap to run, easy to set up and quick to complete, but the animal does not require any training beforehand as the test is unconditioned. The test also has face validity (defined as the ability of a task to visually appear to measure what it is intended to measure; Walf & Frye, 2007). As the test aims to examine behaviour in a more exposed compared to more protected areas, the tests succeeds in demonstrating face validity. In addition to this, as the test is widely used, the researcher is provided with a wealth of background information on how the test may be used, how elements of the methodology may affect results (for example, pre-test handling) as well as there being a wealth of results to compare findings with. As mentioned above, it is not clear that the OF is solely measuring anxiety-like behaviour. The number of entries and duration spent in the centre of the OF could reflect different exploration strategies between the sexes, a search for escape, or a combination thereof, given that

exploration and anxiety-like behaviours can be independent factors (Greenberg 2003). This appears to be supported by the fact that the stress hormone corticosterone is higher in female than male rats when exposed to this and similar tests. The sex difference in locomotion may also reflect a different strategy to approaching a novel space between the sexes (e.g. Lynn & Brown, 2009).

In terms of construct validity (whether an observable variable such as time and entries into the centre measures an unobservable experience such as 'anxiety'; Walf & Frye, 2007), it is not clear whether the OF is fully valid. While pharmacological tests have shown that some anxiolytic and anxiogenic compounds do affect centre square activity in the predicted directions, this result is by no means consistent across all drugs which are known to affect anxiety levels in humans (Prut & Belzing, 2003). This test has also only been behaviourally, physiologically and pharmacologically validated in male rats (Palanza, 2001), which may further suggest that the test could measure different factors in female rats. What is clear however, is that there is a sex difference in behaviour on this test regardless of how it is interpreted (with females locomoting and exploring the centre more than males), and this behaviour has been examined in preclinical research studies to help develop new anxiolytic compounds. Therefore the sex and age differences in behaviour on this test remain of interest, as do the mechanisms for these differences.

6.2 The Emergence Test (ET)

The emergence test has been used as early as 1940 (Anderson, 1940) and consists of a cage, canopy or other type of shelter which is placed inside a novel area that resembles the O.F (Grewel, Shepherd, Bill, Fletcher & Dourish, 1997). The animal is then placed in the relatively safe shelter and the time taken to emerge from this shelter and spent outside of it are measured. The configuration of the test does vary however: a cage resembling the homecage of the rat can be attached to the side of the arena. Alternatively, a rat can begin the test in a start box or under a canopy that is already in the field; these in turn can be placed either along a wall, in a corner, or in the centre square of the

apparatus (see Archer, 1975 for a brief summary of emergence test studies). A diagram of the apparatus as used by our laboratory is provided below in **Figure 1.2**.

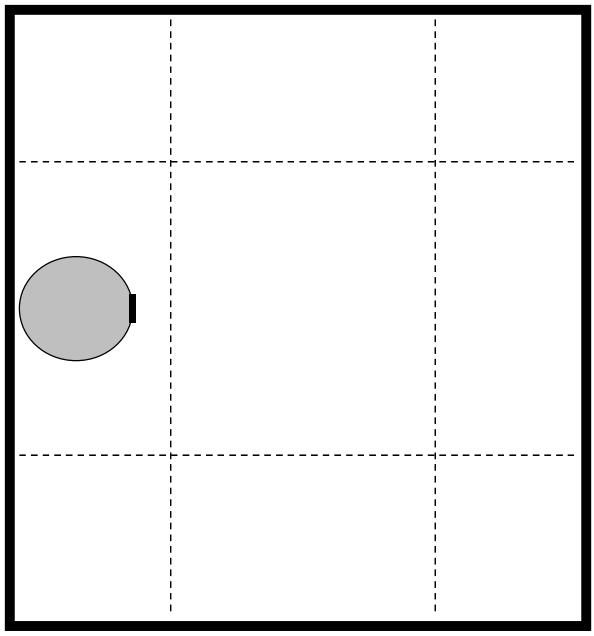

Figure 1.2 Line drawing of the ET as used in our laboratory. The entrance to the start box is indicated by the black line.

Commonly across all configurations of the ET however, animals that emerge quickly (in other words, those that have a low exit latency) and spend less time in their shelter have been considered to exhibit less anxiety-like behaviour than those that take longer to first exit their shelter and spend more of their time there. As the emergence test is less commonly used than the OF, fewer experiments have focused on its pharmacological validation. The research does however support that anxiolytic medications tend to decrease the latency to exit the start box, while anxiogenic compounds increase this latency (e.g. Gallate, Morley, Ambermoon & McGregor, 2002).

Research has typically demonstrated that adult female rodents are faster to exit the start box of the ET than males (e.g. Palanza, 2001), though sex differences are not always found (e.g. Bartolomucci et al., 2004). Less work has been done using the ET with adolescent rats, although one study has shown that adolescent rodents explore less outside of the start cage than adults

despite no significant differences in latency to exit this start cage with age (Arakawa, 2005). As with the OF, even less work has examined differences between the two sexes during this period of life, and we cannot currently find any publications examining sex differences in adolescent behaviour on the ET; more research is therefore warranted.

One of the strengths of the ET is the fact that, in contrast to the OF, the ET is not a 'forced' test. In other words, the animal is not forced to remain in the large, more stressful part of the apparatus; instead, the rat can choose to enter and remain in the relative shelter and safety of a start box or homecage. Similarly to the OF, the test is unconditioned so in addition to not requiring training, it is quick, inexpensive and simple to run. Face validity also holds, in that the test appears to measure the activity of a rat in a novel environment outside of a relatively sheltered start box. Unfortunately however due to the differences in its configuration, making comparisons across the literature is more difficult.

Regarding limitations of the ET, the construct validity is questionable, much like the OF test. Researchers cannot be sure that the measures produced (mainly the latency to first exit the start cage) bear relevance to anxiety due to the lack of pharmacological validation with anxiety-affecting compounds: this work is neither extensive nor consistent across compounds. A short latency to exit may not indicate a low anxiety-like behaviour, but increased exploration or search for an escape route. This latency variable is also easily skewed by extreme values (such as rats never entering the surrounding field). There is however still a sex difference in this behaviour, and with such big gaps in the adolescent literature alone, more work is therefore warranted.

6.3 The Elevated Plus-Maze (EPM)

The elevated plus-maze was developed from Montgomery's elevated alleyway apparatus (1955) and is one of the most common tests of anxiety-like behaviour used by behavioural pharmacologists (Walf & Frye, 2007). The EPM consists of two open arms and two arms enclosed by walls, arranged in a plus-shape and

raised above the ground (Handley & Mithani, 1984; Montgomery, 1955). A diagram of this apparatus is available below in **Figure 1.3**.

On the EPM rodents that spend more time and enter the open arms more than others are considered by some researchers to be exhibiting lower anxiety-like behaviour, while animals that spend relatively more time in the closed arms are considered to show higher anxiety-like behaviour (Walf & Frye, 2007). This test has also been validated pharmacologically, with anxiolytics from the benzodiazepine (BDZ) family well known to increase open arm entries and open time relative to controls (e.g. Pellow, Chopin, File & Briley, 1985). In corollary, some anxiogenic drugs such as caffeine have been found to decrease open arm activity relative to controls (e.g. Pellow et al, 1985; Pellow & File, 1986).

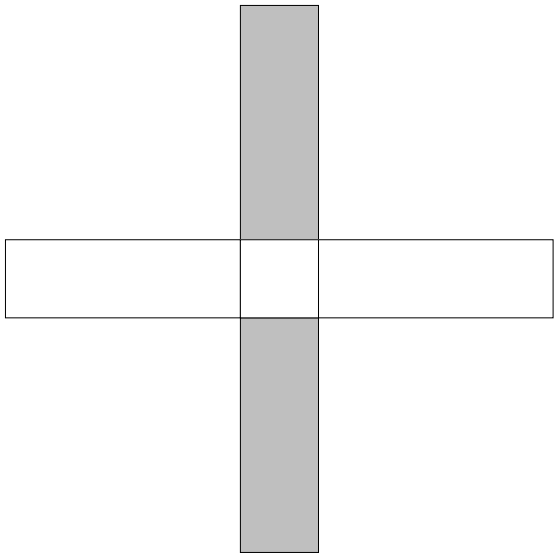

Figure 1.3 Line diagram of the overhead view of the EPM. Grey represents the closed arms, while the open arms and central square are white.

Research examining the behaviour of adult rats on the EPM has consistently found females to enter and remain on the open arms more than male rats (e.g. Aguilar et al., 2003; Johnston & File, 1991; Zimmerberg & Farley, 1993). Experiments examining adolescent behaviour on the EPM have

shown that adolescents spend more time on or enter the open arms more often than adults (Macri, Adriani, Chiarotti & Laviola, 2002; Doremus-Fitzwater, Varlinskaya & Spear, 2009) whilst others have found the opposite (Doremus, Brunell, Varlinskaya & Spear 2003). Regarding sex differences in adolescent behaviour, some experiments have found mid-adolescent females to show less anxiety-like behaviour than same age males on this test (Imhof, Coelho, Schmitt, Morato & Carobrez, 1993; Elliott, Faraday, Phillips & Grunberg, 2004; Leussis & Andersen, 2008), while others suggest that sex differences do not emerge until early adulthood (Estanislau & Morato, 2006). More work is required on when this sex difference emerges, and how adolescent and adult behaviour compares.

As an unconditioned test of anxiety (like the OF and ET), the EPM has the benefit of being simple, quick and inexpensive to run. This test is also the most widely used and validated of these three behavioural tests, meaning that there is a richer and larger literature to compare data with. Also similar to the ET and OF, the EPM suffers from issues of validity and interpretation. The issue of whether open arm activity represents anxiety, exploration, the search for an escape route or a combination thereof remains. With regards to construct validity, not all pharmacological validation studies have supported the idea that this test measure anxiety-like behaviour. For example, Pellow and colleagues (1985) report that while the EPM is successfully validated by the use of the BDZs with which it was designed, this is not the case for other drug families that successfully decrease anxiety in humans (e.g. Borsini, Podhorna & Marazziti, 2002). In fact, the EPM was pharmacologically validated using only male rats, meaning that the test may even tap into different constructs for males and females (Palanza, 2001). This is supported by the behaviour of male and female rats loading differently in factor analyses of EPM behaviour (e.g. Fernandes, Gonzalez, Wilson & File, 1999). Furthermore, the relatively higher corticosterone (stress hormone) response of females in the EPM as well as their higher locomotion than males may also indicate that the exploration strategies between the two sexes are different (e.g. Wigger & Neumann, 1999; Johnston & File, 1991).

6.4 Translational Validity of the OF, ET and EPM

Translational validity is essentially whether the measure assessed using an animal is the same or analogous to that measured in the human research with which it converges (Pryce & Seifritz, 2011). One of the most immediate differences that is apparent between the human and animal literatures on anxiety is the direction of the sex difference: in humans, women more commonly suffer from anxiety than men, while on all three of the behavioural tests reviewed above, adult female rats apparently exhibit lower anxiety-like behaviour than adult males. This may not be as big a problem as it first appears however for two main reasons. First of all, as mentioned above, these tests may actually measure something slightly different in male compared to female rats. While this makes comparing sexes problematic, the ability to examine, for example, the effects of gonadal hormones on behaviour can still be informative by making intra-sex rather than inter-sex comparisons.

Secondly, and perhaps more importantly, the interpretation of the behaviours measured as being 'anxiety-like' is problematic at best, and at worse may be inaccurate. Discriminating between the time spent on the open arms of the EPM for example, as an act of low anxiety-like behaviour, of exploration or of seeking an escape is a difficult one, and in fact, the behaviour may be a balancing act of neophobic and neophilic tendencies (Greenberg, 2003). The fact that the tests are not consistently valid with all known anxiolytic and anxiogenic compounds may be related to this fact. Regardless of the interpretation of the behaviour however, the behaviours measured by these tests do remain consistently dimorphic and are related both to each other (i.e. have predictive validity: for example the percentage entries onto the open arms of the EPM and centre of the OF correlate; e.g. Frye, Petralia & Rhodes, 2000) and to the pharmacological treatment of anxiety. These behaviours thus remain of interest both in their own right within the rodent field of study, and in regards to human anxiety research.

C. Summary of Upcoming Chapters

The following chapters deal with experiments carried out as part of this thesis to examine the importance of adolescence in the emergence of anxiety, both in the rat (chapters 2-4) and in humans (chapter 5).

Chapter 2 details an experiment where the potential effects of the female rat's reproductive cycle on EPM performance were examined. This was to assess whether the oestrous cycle of the female rat needed to be taken into account in the experimental work to follow.

Chapter 3 reports an experiment where rats were tested either at mid-adolescence, late adolescence, early adulthood or later adulthood in the OF, ET and EPM. The aim was to examine whether adolescents and adults exhibit different levels of exploratory behaviour, when sex differences in such behaviours appear, and to see if any patterns in these behaviours emerge as age increases.

Chapter 4 reports an experiment that examined the effects of both testing order and running multiple tests on adolescent compared to adult rats using the EPM and the locomotor box (LB) – a measure of ambulation. The aim was to examine the sensitivity of EPM performance to prior novel test experience, and to see if rats of different ages or sexes are affected differently by the order of testing, as this is an important factor to consider when comparing adult and adolescent , or male and female behaviour across multiple tests.

In Chapter 5, interpretive bias was examined using a questionnaire measure in adolescent boys and girls. Sex differences in this bias and its relationship to pubertal status and anxiety were examined, given that interpretive bias is a potential cognitive vulnerability to anxiety, and puberty may be an important event in the emergence of anxiety.

This thesis then concludes by summarising the outcomes of these experiments, and where the research should be focused next in order to address the questions raised throughout the thesis (Chapter 6).

Chapter 2: The Effects of the Oestrous Cycle on Elevated Plus-Maze and Locomotor Box Performance in Lister-hooded Rats

1. Introduction

Gonadal hormones naturally vary in intact female rats as part of their ovarian cycle, known as the oestrous cycle, and may affect anxiety-related behaviour as have laboratory-controlled manipulations of female gonadal hormones (e.g. Bitran Purdy & Kellogg, 1993; Frye & Walf, 2004; Hill, Karacabeyli & Gorzalka., 2007; Zimmberg & Farley, 1993). This experiment aimed to examine whether EPM and locomotor box (LB) performance differed across phases of the oestrous cycle in our laboratory's strain of rat, the Lister-hooded, and how this compared to adult male rats.

The rat's oestrous cycle lasts 4-5 days and is commonly divided into 4 phases (Becker et al., 2005): *diestrus I* and *II* (during which both estradiol7 and progesterone levels are relatively low), *proestrus* (during which estradiol levels peak and progesterone levels begin to rise), and *oestrous* (during which estradiol levels are low and progesterone levels peak). These stages of the cycle are easily determined by examining epithelial cell samples collected from the vagina of the rat in a flushing process known as lavage (**Figure 2.1**). In the wild, a variety of behaviours are known to occur with different phases of the cycle. For example, on the evening of proestrus and morning of oestrous, female rats are sexually receptive and exhibit solicitational behaviour, such as hopping, darting and ear wiggling (Erskine, 1989; McCarthy & Becker, 2002). In the wild, female rats will wander beyond the normal limits of their home ranges during the oestrous phase, leaving scent marks that potentially advertise their location and receptiveness to males (Calhoun, 1963).

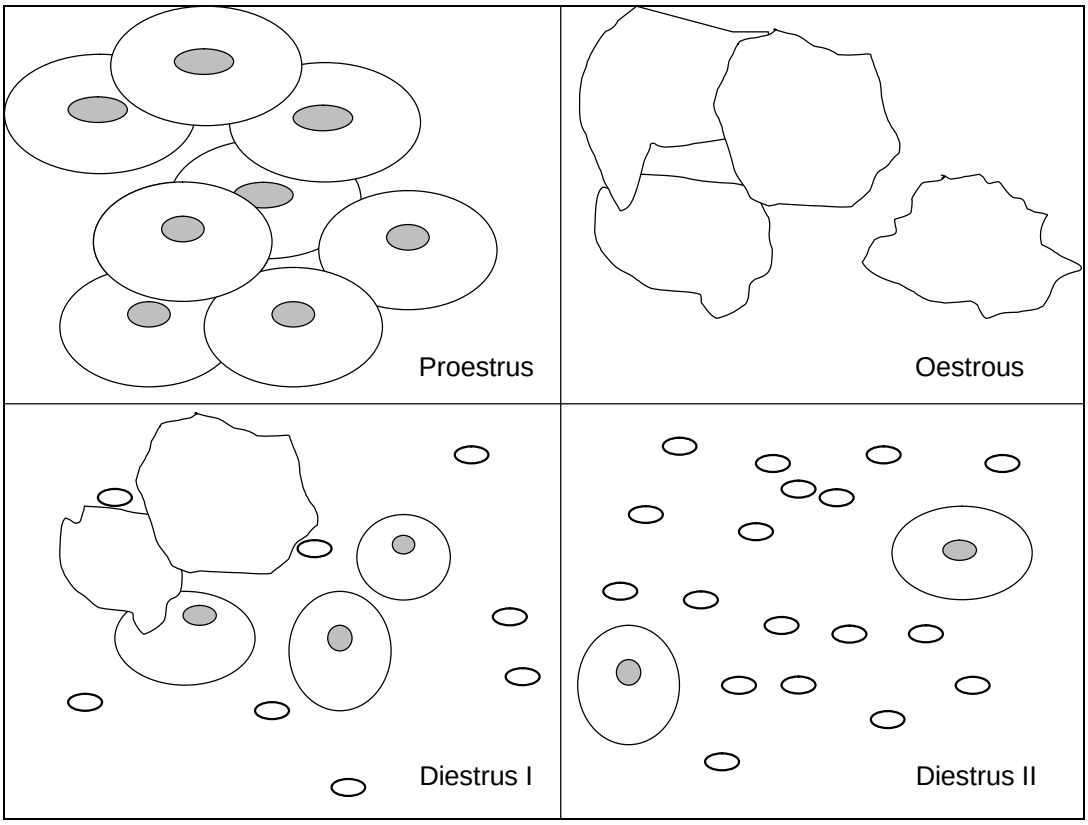

Figure 2.1: schematic diagram of cell types found at each stage of the oestrous cycle.

Due to the increased ranging and exploring that occurs in wild female rats, it stands to reason that related behaviours may be recorded in the laboratory rat. In line with this, an increase in wheel-running and general locomotor behaviour has also been reported in rats during proestrus and early oestrous phases of the cycle (e.g. Anantharaman-Barr & Decombaz, 1989; Archer, 1975; Frye et al., 2000; Petersson et al., 1998; Richter, 1927; Schneider & Popik, 2007; Wang, 1923). The EPM has also been used in the examination of behaviour changes across the cycle; female rats have been reported to spend more time on the open arm of the EPM when circulating estradiol and progesterone levels are high (i.e. during proestrus and early oestrous) than at other stages of the cycle (e.g. Bitran & Dowd, 1996; Diaz-Veliz, Alarcón, Espinoza, Dussaubat, & Mora, 1997; Frye et al., 2000; Marcondes, Miguel, Melo & Spadari-Bratfisch,

2001; Molina-Hernández, Olivera-Lopez, Tellez-Alcántara, Pérez-García & Jaramillo, 2006; Mora, Dussaubat & Diaz-Veliz, 1996).

However, other studies examining behaviour across the oestrous cycle have failed to find higher levels of open arm activity in proestrus female rodents compared to females at other stages of the cycle (e.g. Bitran, Hilvers & Kelogg, 1991; Fernández-Guasti, Martínez -Mota, Estrada-Camarena, Contreras & López-Rubalcava, 1999; Nomikos & Spyraki, 1988; Sadeghipour et al., 2007; Schneider & Popik, 2007), while locomotor studies have been more consistent in reporting heightened activity in proestrus and oestrous females (e.g. Palanza, Gioiosa & Parmigiani, 2001; Sell, Thomas & Cunningham, 2002; Steiner, Katz, Baldrighi & Carroll, 1981; Wang, 1923) One study has even reported opposing results; Sadeghipour and colleagues (2007) found that females in diestrus I, not proestrus, spend more time on the open arms of the EPM than ovariectomised (OVX) females. There are several methodological reasons for these inconsistencies however, as will be reviewed in the chapter discussion, including that fact that EPM behaviour differs between rodent strains (e.g. Ramos, Berton, Mormède & Chaouloff, 1997; van der Staay, Schuurman, van Reenen & Korte, 2009). We have not found any published experiments that involve the pigmented strain of Lister-hooded rat, a strain that is used in our lab and across Europe for visual and cognitive experiments (McDermott & Kelly, 2008).

To examine the effects of the oestrous cycle on locomotor and EPM behaviours, adult female Lister-hooded rats performed a 5 minute EPM and a 5 minute LB test during either their proestrus, oestrous or diestrus cycle phase. Males were also included for comparison, receiving lavage-like handling. Using standard experimental conditions that we planned to implement in the experiments to follow, we wanted to determine whether stage of the oestrous cycle needed to be taken into account in future experiments. As the EPM relies on novelty (e.g. it is sensitive to pre-test manipulations and multiple exposures; e.g. Dawson, Crawford, Stanhope, Iversen & Tricklebank, 1994; Doremus-Fitzwater, Varlinskaya & Spear, 2009), the EPM was always the first novel environment to which the animals were exposed. Given that both wild and

laboratory female rats have been found to locomote and explore more during late proestrus and early oestrous, we predicted that females in these cycle phases may be more locomotory on both pieces of apparatus, and enter or remain in the open arms more so than diestrus females.

2. Material and Methods

2.1 Subjects and Housing

The subjects were 50 adult Lister-hooded rats (40 females and 10 males; supplied by Harlan, U.K.). Upon arrival, the females weighed 156-211g and the males weighed between 253-284g. The animals were housed in same-sex pairs in plastic and wire mesh homecages (52 x 40 x 26 cm) in the same holding room (temperature 20 ± 1ºC; relative humidity: 55 ± 5%; lights on from 07:00 – 19:00; light level of 90 lux). Water and soy-free pelleted food were available *ad libitum*. At the beginning of the experiment, the females weighed 172-229g and the males 273-318g. All animals were treated in accordance with the Animals (Scientific Procedures) Act 1986 and regulations set by the University's internal Animal Welfare Ethics Committee.

2.2 Determination of Ovarian Cycle Stage

Ovarian cycle stage was determined by examining epithelial cells from the vagina: these were obtained via a vaginal flush technique known as lavage. To obtain the smear sample, the animal was gently wrapped in a cloth to restrict movement, and a sterile pipette containing saline was inserted into the vagina. The saline was flushed out of the pipette, re-collected, and placed onto a glass microscope slide.

A light microscope was used to examine the slides within 30 minutes of lavaging. Diestrus I was characterised by a mixture of cornified cells, nucleated cells, and leucocytes; diestrus II was characterised by numerous leucocytes, few nucleated cells, and no cornified cells; proestrus was characterized by nucleated cells and occasional cornified cells; oestrous was characterized by clusters of cornified cells and a lack of leucocytes (Long & Evans, 1922; Becker et al., 2005). Given that diestrus I can be difficult to confirm due to the relatively

short period of this stage, subjects were selected for behavioural testing at only three of the stages: diestrus II (referred to as diestrus from here onwards), proestrus and oestrous. **Figure 2.2** shows photographs of typical smear samples obtained during this experiment. All females were lavaged between 09:50 and 11:00 hours for ten consecutive days and only those females exhibiting two regular four or five day cycles were included. Males were subject to the same handling procedure as females each day.

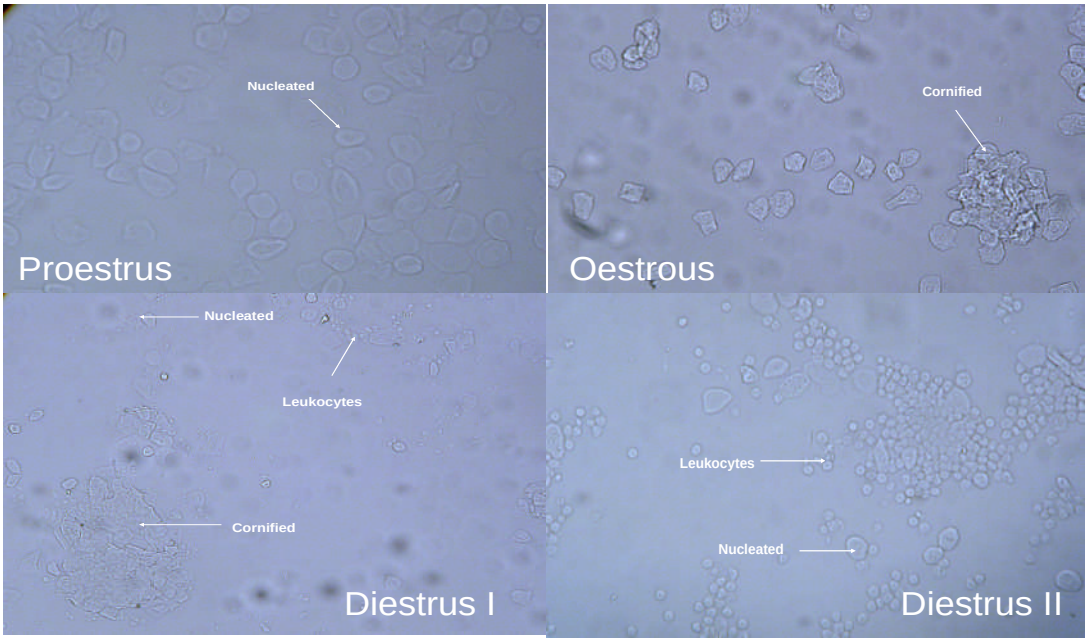

Figure 2.2 Photographs of typical vaginal smears taken with a light microscope as part of this experiment during each phase of the oestrous cycle. Different cell types that characterise each phase are labelled.

On the five days following the period of lavaging, female subjects were selected for behavioural testing based on their predicted cycle phase, which was confirmed by lavage on the test day (at least 2 hours before behavioural testing). The final sample size for behavioural testing was 29 females (8 proestrus, 12 oestrous and 9 diestrus) and 10 males.

2.3 Confirmation of Ovarian Cycle Stage Using an Estradiol Assay

In order to confirm that our method for assigning females to particular cycle stages was accurate, blood samples were collected from 24 female subjects after completion of the behavioural testing to be analysed using an estradiol assay. The females were lavaged in the morning between 9:50 and 11:00 as per behavioural testing, and 8 subjects were selected for blood sampling at each of the three cycle stages: diestrus, proestrus and oestrous. Each subject was anaesthetised using isoflurane gas. Once unconscious, the animal's tails were washed with warm water and cotton, then disinfected with an alcohol wipe. Using a sterile needle, a blood sample (1ml) was taken from the lateral tail vein. Pressure was applied to stop the vein bleeding after the sample was collected, and the animals were allowed to regain consciousness in a heated recovery cage before being returned to their homecage. All samples were collected between 13:00-16:00 hours and allowed to clot at room temperature overnight before being centrifuged at 3,000 rpm for 10 minutes. The serum was then pippetted off and frozen (-80°C) for later analysis.

An enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine serum estradiol-17*β* levels, using polyclonal (rabbit) antibody-coated wells (EIA-2693, DRG Instruments GmbH, Germany). The sensitivity of the kit, defined as the lowest detectable level that can be distinguished from *Standard 0*, was 9.714 pg/ml. Intra-assay variability was 4.66% and inter-assay variability was 7.79%.

2.4 Apparatus

2.4.1 EPM

The EPM consisted of four painted, wooden arms (51cm long x 11cm wide) raised 56cm from the ground on a metal frame (see **Figure 2.3**). Two of the arms had wooden walls (closed arms; 40cm high) and the remaining two arms lacked walls (open arms). The central area was 11cm^2.

Figure 2.3: a photograph of the EPM used in our laboratory.

2.4.2 LB

The locomotor box consisted of a transparent, perspex box (46cm long x 24cm wide x 22cm high; LEDREARING model, Hamilton-Kinder, LLC., USA) that was surrounded by a set of photo-beams. Breaks in the photo-beams were automatically recorded by a computer running MotorMonitor software (version 4.14, Hamilton-Kinder, LLC, USA). The set of six locomotor boxes were in a small testing room, separate from the EPM testing room (**Figure 2.4**).). Animals completed a 5 minute test, at least one hour after the E.P.M.

Figure 2.4: a photograph of the locomotor boxes in our laboratory.

2.5 Experimental Design

The animals were lavaged for ten consecutive days, and the five days following this lavaging period constituted the test period: females were selected on their predicted cycle phase, which was confirmed by lavage on test day, at least two hours before the EPM. Due to the number of subjects, the animals were lavaged and tested in two batches, thus the whole experiment occurred over a period of 30 days. Males were subject to the same handling that females had received during lavage, and according to the same schedule. The order was randomised so that one sex was not always handled first. Lavaging and testing always occurred during the light phase of the cycle, as the animal house was not equipped for reverse-light schedules due to no double door entry to block out light from the corridor. Light phase testing was used across two thirds of the relevant studies reported in this chapter (**Table 2.1**, page 66).

The animals' first test was the EPM. This occurred between 13:00 – 16:00. The subject was carried from the housing room to the testing room in a

small carry box with a solid lid (45 x 28 x 13 cm), then placed in the centre of the EPM facing a closed arm. Each test lasted 5 minutes and was carried out in dim white light (approximately 27 lux). Each trial was filmed with an overhead digital camera, with the apparatus behind a black curtain meaning that the observer was not visible to the subject. The feed was played live on a computer in the same room as the apparatus, and behaviours were recorded using software developed and created within the School of Psychology, University of St Andrews. The apparatus was cleaned with 70% alcohol solution after each subject and allowed to dry.

The subjects' second test was the locomotor box, occurring between 14:00 – 17:00. The animals had at least a one hour break between the EPM and this test. Each animal was placed in a locomotor box, the lid was put into place and the 5 minute test began, with the observer leaving the room until the trial had elapsed. Up to 4 animals performed the test at any one time. The apparatus was cleaned with a 70% alcohol solution and allowed to dry between each subject.

2.6 Behavioural Measurements

2.6.1 EPM

In the EPM, the following behaviour patterns were recorded:

a) *enter a new arm or central area*: all four of the animal's paws cross the boundary into a different arm or into the central area.

b) *peek onto an open arm*: the animal puts its head out onto an open arm, at least up to the level of the ears, without entering it.

Total locomotion was defined as the total number of entries into closed and open arms and the central area. *Percentage of entries* into each area were calculated as the number of entries into that area divided by the total locomotion, multiplied by 100. *Percentage of time* spent in a particular area of the apparatus was calculated by dividing the time spent in the area by the total duration of the test, multiplied by 100.

2.6.2 Locomotor Box

In the LB, the following activities were recorded:

a) *basic movements*: an animal's body breaks a photobeam, while another simultaneously reforms i.e. these are the whole body movements.

b) *fine movements*: an animal's body breaks a photobeam *without* another becoming reforming; these movements may include rearing, sniffing and grooming.

From these measures, the total number of basic movements and fine movements over the five minute test period were calculated.

2.7 Statistical Analyses

As a high number of subjects did not have detectable levels of estradiol, a chi squared test was performed to compare the number of animals with detectable/undetectable levels of estradiol in each phase of the oestrous cycle. The EPM data were tested for normality using the Shapiro-Wilk test, and variables that were not normally distributed were log transformed. Normal and normalized data were analysed using multivariate analyses of variance (MANOVAs) in SPSS (version 14). The between-subjects factor was 'group' (diestrus, proestrus, oestrous female and male) and the within-subjects factors were the recorded behaviour patterns. To analyse arm preference, the percentage durations were run in a separate repeated measures ANOVA separated by subject group, with arm as the repeated-subjects measure. The LB data were analysed in the same way using a separate multivariate ANOVA, with group as the between-subjects factor and the behaviours as within-subject factors. *Post-hoc* comparisons (Scheffe's test) were performed where appropriate. An alpha level of $\alpha < .05$ was used throughout. Data reported in brackets are means ± SEMs. Effect sizes are reported as η_p^2.

3. Results

3.1 Estradiol Assay

There was a significant difference in cycle phases for estradiol detection ($\chi^2_2 = 10.53$, $p = .005$), resulting from the fact that only those animals characterized by

microscopy as being in proestrus had measurable levels of estradiol. These results therefore support the categorization of cycle phase via lavage and microscopy.

3.2 EPM

3.2.1 Total Open and Closed Arm Entries

There was a main effect of group on total open arm entries ($F_{3,35}$ = 3.06, p =.042; η_p^2 = .218). *Post-hoc* tests revealed that diestrus females made more open arm entries than males (see **Figure 2.5**). There was no main effect of group on total closed arm entries ($F_{3,35}$ = 0.32, p = .808; η_p^2 = .029).

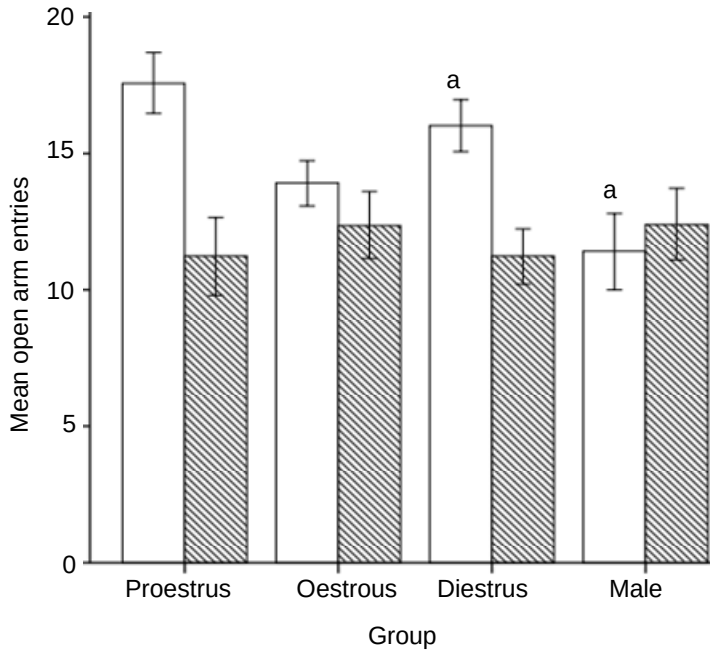

Figure 2.5: total entries onto the open (white bars) and closed arms (hatched bars) of the EPM. 'a' represents a significant post-hoc difference at p < .05.

3.2.2 Percentage Open and Closed Arm Entries

There were no significant differences amongst the groups of rats in terms of the percentage of entries made onto the open arms of the EPM ($F_{3,35}$ = 2.31, p = .093, η_p^2 = .165). There were also no significant differences between the groups

in terms of the percentage of closed arm entries ($F_{3,35}$ = 2.06. p = .123, η_p^2 = .150).

3.2.3 Percentage Open and Closed Arm Durations

Group had a significant main effect on the percentage of time spent on the open arms ($F_{3,35}$ = 4.71, p = .007; η_p^2 = .295). *Post-hoc* tests revealed that there were no cycle effects, although diestrus females were spending a greater percentage of their time there than male rats (**Figure 2.6**). More importantly however, females in each cycle phase spent significantly more time on the open than closed arms, while males showed no preference for either arm type (**Figure 2.6**).

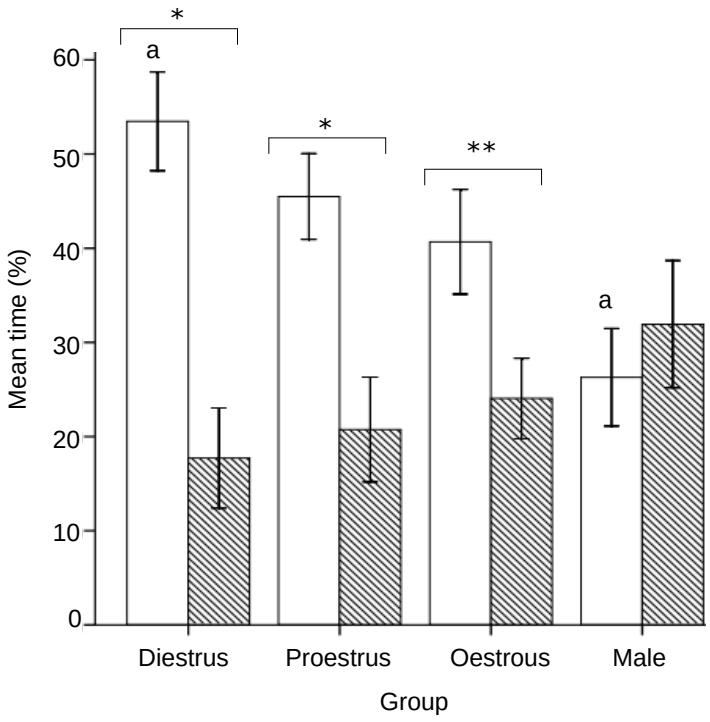

Figure 2.6: Mean percentage duration on the open arms (white bars) and closed arms (hatched bars) of the EPM. 'a' represents a significant post-hoc comparison at p < .05. * indicates a significant main effect of arm at p < .05, ** at p < .01.

3.2.4 Peeking

There was a significant main effect of group on peeking from the centre of the EPM ($F_{3,35}$ = 4.70, p = .008; η_p^2 = .300), with males peeking more than diestrus (p = .024) and oestrous females (p = 0.34; proestrus = 6.3 ± 0.9; oestrous = 5.5 ± 0.8; diestrus = 4.7 ± 0.9; male = 8.0 ± 0.8).

3.2 Locomotor Box

There was a main effect of group on basic movements ($F_{3,35}$ = 10.45, p < .001, η_p^2 = .468). The *post-hocs* revealed that female rats in each phase of the cycle ambulated significantly more than the males (**Figure 2.7**). There was also a main effect of group on fine movements ($F_{3,35}$ = 4.27, p = .011, η_p^2 =.276), though only diestrus females made more fine movements than males (**Figure 2.7**).

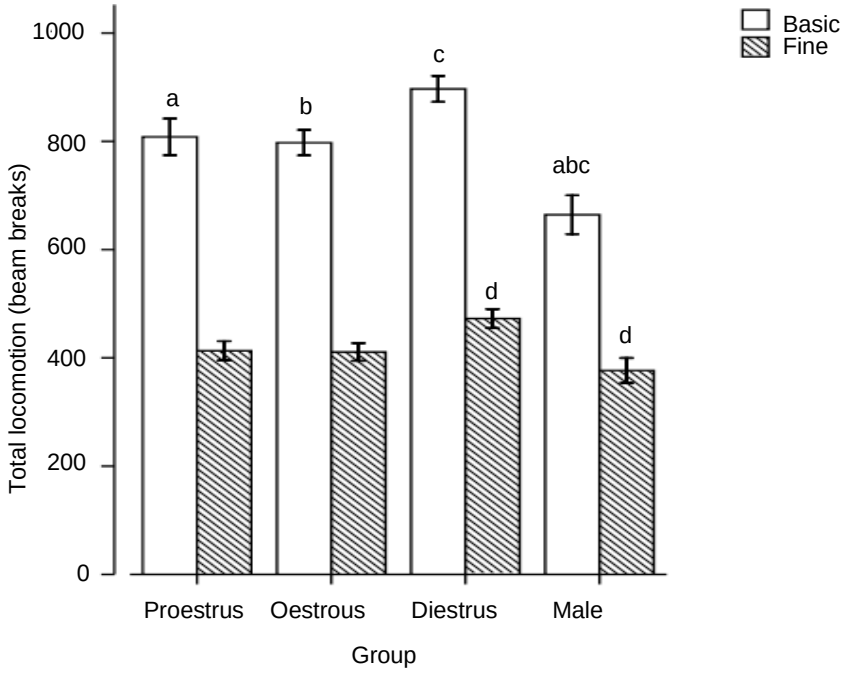

Figure 2.7: mean basic (white bars) and fine (hatched bars) locomotion in the LB. Identical pairs of letters represent significant differences: a represents a significant post-hoc comparison p < .001, b c and d represent a significant comparison at p < .05.

4. Discussion

The aim of this study was to examine whether there are cycle effects on EPM and LB performance in the Lister-hooded strain of laboratory rat. In the EPM, results showed there were no cycle effects on open arm activity. Females in each stage of the cycle spent more time on the open rather than the closed arms, while males showed no arm preference. In the LB, females in each phase of the oestrous cycle made more basic movements than males, but did not significantly differ from one another. Thus this experiment appears to demonstrate that, under the conditions used here, there are no detectable cycle differences in the behaviour of female rats on the EPM and LB, though sex differences between females and males can still be found.

This experiment is in agreement with other work that has reported no effects of the oestrous cycle on EPM behaviour (**Table 2.1**). For instance, Nomikos and Spyraki (1988) found no cycle effects or differences between cycling and ovariectomised (OVX) females in terms of the percentage of open arm entries made or time spent there. Another study has reported finding no cycle effects on any open arm or general arm entry measures (Fernandez-Guasti et al., 1999). The locomotor box results also failed to show any cycle differences in ambulation in this experiment, although all females made more basic movements than males. The lack of cycle differences in locomotion is a less common finding than with EPM behaviour, however some studies have found no differences in locomotion according to the phase of the ovarian cycle (e.g. Galeeva & Tuohimaa, 2001; Zuluaga et al., 2005). Sex differences were clearly seen in our experiment however, and females locomoting more than males is a finding robustly reported using a variety of apparatuses (e.g. Blizard, Lippman & Chen, 1975; Brown & Nemes, 2008; Johnston & File, 1991).

The findings of this experiment are however contrary to the work of several other researchers. For instance, Diaz-Veliz and colleagues (1997) found that both proestrus and oestrous females spent more time on the open arms and made a greater percentage of entries onto the open arms than either diestrus I or II females. Frye and colleagues (2000) reported proestrus females to make both more total open and closed arm entries than either oestrous,

diestrus I or diestrus II females. Other studies have found the same or similar results (Bitran & Dowd, 1996; Marcondes et al., 2001; Mora et al., 1996). In terms of locomotion, proestrus and oestrous females have also been found to locomote more either in homecage running wheels, locomotor boxes or other pieces of apparatus that permit ambulation (e.g. Sell, Thomas & Cunningham, 2002; Steiner, Katz, Baldrighi & Carroll, 1981; Wang, 1923). There are some important methodological reasons that may explain why this experiment has found different results to those listed above however, namely the time of the day of testing, sample size and strain of rat examined (see **Table 2.1** for a summary).

Time of day may be important in the detection of ovarian cycle effects on behaviour as the rat's true behavioural oestrous is said to occur at night, around the time of ovulation (Becker et al., 2005). This is the time where, in the wild, the animal is most active in laying scent marks and thus it explores more of its environment (Calhoun, 1963). Therefore, tests such as the EPM and LB may be most likely to detect effects of the oestrous cycle on behaviour when tested during the dark-phase of the rat's light-dark cycle. Certain studies have detected cycle differences on EPM behaviour when testing in the dark (Bitran & Dowd, 1996; Frye et al., 2000). However, studies have reported cycle effects on EPM behaviour in the light phase of the day (e.g. Diaz-Veliz et al., 1997; Marcondes et al, 2001; Mora et al., 1996; Sadeghipour et al, 2007), while testing during the dark phase does not always lead to cycle effects being detectable (e.g. Fernandez-Guasti et al., 1999). Locomotor differences have also been reported more so in dark phases experiments (e.g. Sell, Thomas & Cunningham, 2002; Steiner, Katz, Baldrighi & Carroll, 1981; Wang, 1923) than light phase experiments (e.g. Stoffel & Craft, 2004).

The second methodological issue that may be important is that of sample size. This study had 8-12 animals per group, which is within the common range of sample size used in this field (**Table 2.1**). However, studies using similar sample sizes have reported the presence of cycle differences in EPM behaviour (Diaz-Veliz et al., 1997; Sadeghipour et al., 2007). A larger sample size is also not necessarily sufficient for detecting cycle differences in such behaviours

either, such as Fernandez-Guasti and colleagues' work (1999) where 14-15 animals were included in each cycle phase group. With regards to cycle effects on locomotor behaviour, significant differences have been found with as few as 4-6 animals per group (Sell et al., 2002). In summary, while our sample size should be sufficient for detecting cycle effects, a large sample size alone may not necessarily provide the sensitivity needed to measure fluctuations in behaviour across the cycle.

The final and strongest methodological difference that may account for this study not finding cycle effects is the strain of rat used: while we used Lister-hooded rats, we could find no other study that uses the same strain of rat (**Table 2.1**). Behaviour on the EPM has been shown to differ between different strains of rat (e.g. Ramos et al., 1997; van der Staay et al., 2009), including strain differences in the sensitivity to different EPM-affecting drugs.between Listers and two albino strains (the Wistar and Sprague-Hawley rats; Hogg, 1996). One reason that no difference in open arm behaviours may have been found across the cycle in the Lister-hooded strain of rat is the high baseline levels of open arm activity. In this experiment, female rats spent 40-55% of the time on the more aversive open arms of the EPM; these are in fact levels typical of open arm duration in rats who have received anxiolytic drugs (e.g. Drapier et al., 2007; Stock, Foradori, Ford & Wilson, 2000; Violle et al., 2009).

One other study that failed to find ovarian cycle effects on EPM behaviour (Schneider & Popik, 2007) also reported high percentages of time spent on the open arms in extensively handled subjects (around 45-55% in proestrus females). This suggests a potential ceiling effect, whereby no further alterations in open arm activity could be detected. This fits with other studies that did find ovarian cycle effects on EPM behaviour, where the amount of time on the open arms rose to between 30 and 50% from other cycle phases to their peak in proestrus females (e.g. Bitran & Dowd, 1996; Diaz-Veliz et al., 1997; Frye et al., 2000; Mora et al., 1996). Therefore, because the diestrus females in our study showed such high durations on the open arms of the EPM (45 - 67%), there may have been no room for further increases to be detected. While it could be argued that the levels of open arm duration seen in this study are not

Table 2.1 Summary of the methodologies used by papers looking at cycle effects on EPM behaviour

Paper	Rat Strain	Sample Size	Phases Studied	Light-Dark Phase at Testing (Lavage, EPM)	Cycle Effects
Bitran & Dowd (1996)	Long-Evans (pigmented)	8 animals, within-subjects	Proestrus, Oestrous, Diestrus	Dark phase	Found
Mora et al. (1996)	Sprague-Dawley (albino)	15-18 / group	Proestrus, Oestrous, Metestrus, Diestrus	Light phase	Found
Diaz-Veliz et al. (1997)	Sprague-Dawley (albino)	10 / group	Proestrus, Oestrous, Metestrus, Diestrus	Light phase	Found
Frye et al. (2000)	Long-Evans (pigmented)	19-22 / group	Proestrus, Oestrous, Diestrus	Dark phase	Found
Marcondes et al. (2001)	Wistar (albino)	14-20 / group	Proestrus, Oestrous, Metestrus, Diestrus	Light phase	Found
Sadeghipour et al. (2007)	Sprague-Dawley (albino)	10 / group	Proestrus, Oestrous, Metestrus, Diestrus	Light phase	Found
Nomikos & Spyraki (1988)	Wistar (albino)	8 / group	Proestrus, Oestrous, Metestrus, Diestrus	Unspecified	Not found
Fernandez-Guasti et al. (1999)	Wistar (albino)	14-15 / group	Proestrus, Oestrous, Metestrus, Diestrus	Dark phase	Not found
McCormick et al. (2008)	Long-Evans (pigmented)	24 total (/ group not specified)	Proestrus and Oestrous v. Metestrus and Diestrus	Light phase	Not found
This study	Lister-hooded (pigmented)	8-12/group	Proestrus, Oestrous and Diestrus(-2)	Light phase	Not found

truly baseline, but are high as a product of the lavage handling, as pre-test handling has been found to increase open arm activity (e.g. Hogg, 1996; Schmitt & Hiemke, 1998). However, female and male subjects did not receive more lavage handling than is typical in the protocol reported in other experiments.

The strengths of this study include its sample size, removal of the short period of diestrus I rather than collapsing this with diestrus II, the use of more than one piece of apparatus and the inclusion of adult male rats for comparison. Despite these benefits however, no cycle effects on the EPM and LB performance of female Lister-hooded rats were found in this experiment, although this does not necessarily preclude that the hormones which vary across the ovarian cycle can affect locomotor and EPM behaviour. We postulate that the high baseline levels of EPM behaviour in this study may have prevented any cycle effects from being detected due to a ceiling effect. We conclude that stage of the oestrous cycle does not need to be taken into account in the studies to follow, given that the Lister-hooded strain of rat and light-phase testing will be used.

Chapter 3: The Ontogeny of Emergence Test, Open Field and Elevated Plus-Maze Behaviour across Adolescence and Adulthood in the Lister-hooded Rat

[The publication arising from this chapter is available in **Appendix 1**].

1. Introduction

As reviewed in the general introduction, surprisingly little research has focused on the changes in anxiety-related behaviours in the rat across adolescence and into adulthood, and such research has neglected to examine sex differences. The aim of this study was to examine how performance on common tests of anxiety-like behaviour (the emergence test [ET], open field [OF] and the elevated plus-maze [EPM]) vary across adolescence and into adulthood in male and female Lister-hooded rats.

Previous studies examining adolescent behaviour in the ET, OF and EPM tests have produced inconsistent results. In the OF, adolescent rodents have been reported to exhibit either lower levels (e.g. Candland & Campbell, 1962) or higher levels (e.g. Arakawa, 2005; Bronstein, 1972; Philpot & Wecker, 2008) of locomotor exploration than adults. In the EPM, adolescents have been reported to spend either less time (e.g. Doremus, Brunell, Varlinskaya & Spear 2003) or more time (e.g. Macri, Adriani, Chiarotti & Laviola, 2002; Doremus-Fitzwater, Varlinskaya & Spear, 2009) on the open arms in comparison to adult rats. Less work has been done using the ET, although one study has reported that adolescents spend less time exploring outside of the start cage than adults despite no significant differences in latency to exit this start cage with age (Arakawa, 2005). Other studies have failed to find age differences in performance on these pieces of apparatus (e.g. OF: Arakawa, 2005; EPM: Hefner & Holmes, 2007). However, many of these studies (e.g. Philpot & Wecker, 2008; Slawecki, 2005; Stansfield & Kirstein, 2005) have only examined one adolescent and one adult age group, therefore collapsing together the distinct sub-stages of adolescence and preventing examinations of how behaviour changes across the period of

adolescence itself and into adulthood. Methodological differences between studies are likely to have contributed to the conflicting results, and many studies were limited to the use of only one behavioural test (e.g. Doremus, Varlinskaya & Spear, 2007; Doremus-Fitzwater *et al*., 2009a; Slawecki, 2005). Conclusions about the changes in anxiety-like behaviour are not easily extrapolated from one behavioural test: for reliability and validity, more tests are required, with correlations between measures from different tests helping to support any conclusions about general changes in exploratory behaviour at this age.

Previous work in our laboratory has compared the behaviour of early (pnd 21-33), mid (pnd 34-46) and late adolescent (pnd 47-59) Lister-hooded rats on two tests of anxiety-related behaviour: the OF and EPM (Lynn & Brown, 2009). The animals (7-9 rats per age x sex subgroup) were tested first on the OF and secondly on the EPM, with a one week period between tests. In the OF, there were main effects of sex and age on total locomotion, with females making more line crossings than males and late adolescents making more line crossings than early adolescents. While there was no interaction, both these effects appeared to be driven by an increased level of locomotion in the late adolescent female rats. There was also a main effect of age on the percentage of entries into the centre of the OF, with older animals making more entries than younger animals. Both these main effects of age persisted when body weight was added as a co-variate to control for differences in body size. In the EPM, there was a main effect of sex on total locomotion, with females locomoting more than males, while total locomotion tended to be greater in older animals. Main effects of sex and age were found for the percentage of time spent on the open arms however, with females spending more time on the open arm than males, and late adolescents spending more time there than both early and mid-adolescent rats. This main effect of age was not eliminated once body weight was controlled for. Correlation analyses also revealed a significant positive correlation between OF and EPM total locomotion, while the percentage of centre entries in the OF tended to correlate positively with the percentage of entries onto the open arms of the EPM. Overall sex differences were already detectable using these groups of adolescent rats. Although age and sex did not significantly interact, the results

revealed that these sex differences mainly resulted from the high locomotion and open arm activity of late adolescent female rats.

This Lynn and Brown (2009) study had the benefit over previous studies of using three time points across adolescence, it involved both sexes of animal, and used multiple pieces of apparatus to assess behaviour rather than drawing conclusions from one test. The question of whether adolescent rats' exploration of novel pieces of apparatus is greater, lesser or similar to adult rodents requires the addition of adult groups for comparison with adolescents however. Many other studies have only compared one adolescent age group to adult rats (e.g. Philpot & Wecker, 2008; Slawecki, 2005; Stansfield & Kirstein, 2005), preventing general patterns across development from being assessed.

The aim of the current experiment was to build on the Lynn and Brown (2009) study by comparing the behaviour of adolescent to adult rats by examining behaviour at four, more narrowly defined age groups: mid-adolescence, late adolescence, young adulthood and older adulthood in a between-subjects design. A compressed testing schedule was used in order to limit the age range of the rats involved (c.f. one week period between tests in Lynn & Brown, 2009), meaning that the mid- and late adolescent groups in particular were clearly distinguished. The animals' behaviour was examined on the ET, OF and EPM, using 24hr breaks between tests. Both male and female rats were included at each age group to examine sex differences. Given that body weight varies between these age groups and animals of different sizes may, for example, differ in their ability to move around and their levels of fatigue, this was measured and controlled to ensure that differences in locomotion and area entries are not a product of differences in body size. This was done in place of scaling the apparatus for the crown-to-rump length of each age group as it is not clear that the animal's perception of their surroundings scales in the same way, and due to the fact that wild adolescent rats explore the same burrows and paths as adult rats do (Calhoun, 1963). Early adolescents were not included in this study as Lynn and Brown (2009) found them to exhibit reliably low levels of exploration compared to late adolescents, already supporting an increase in exploration across adolescence itself.

Following on from the findings of Lynn and Brown (2009), it was hypothesised that locomotion in each test would increase across the age groups, and be higher in males than in females. In the ET, there may also be main effects of age and sex for the latency to exit the box and the number of re-entries into it, with females being faster to exit the start box than males and making fewer re-entries, and with latency and re-entries decreasing across the age groups. While adult and adolescent behaviour is anticipated to differ, it was not known whether adolescents would be more or less exploratory than adults. In the OF and EPM, female rats were hypothesised to make more entries and spend more time in the centre square and open arms of each apparatus. These measures were also predicted to increase across the adolescent groups, although adult levels of exploration could be either higher or lower than adolescents.

2. Materials and Methods

2.1 Subjects and Housing

The subjects were 72 (35 male, 37 female) Lister-hooded rats (*Rattus norvegicus*) selected from nine litters bred in-house (breeding stock acquired from Harlan, U.K.). The breeders and offspring were housed in a holding room (lights on 07:00-19:00; temperature: 20 ± 1ºC; relative humidity: 55 ± 5 %; light level of 90 lux) in opaque plastic and wire mesh home cages (52 x 40 x 26 cm). Water and soy-free pelleted food were available *ad libitum*. The subjects were weaned from their dam and placed into same-sex groups at pnd 21. At pnd 29, the animals were then rehoused into same-sex sibling pairs or triplets.

Separate groups of animals were used in each age category, as prior exposure to a novel apparatus has been shown to influence later performance (e.g. Bertoglio & Carobrez, 2000). The following numbers of subjects were allocated to each age category: mid-adolescence (pnd 34-39) = 9 males, 10 females; late adolescence (pnd 51-55) = 9 males, 8 females; young adulthood (pnd 65-69) = 9 males, 10 females; and older adulthood (pnd 104-109) = 8 males, 9 females (for weights, see **Table 3.1**). No more than two subjects in each age group were taken from a single litter. Each animal was handled only

for the purpose of transporting between the holding room and testing room, and for weekly weighing and cage cleaning.

Table 3.1 Mean weights (g) and SEMs of each age and sex sub-group of animals.

	Males (g)	Females (g)
Mid-adolescence	126.1 ± 15.1	107.7 ± 14.3
Late adolescence	217.2 ± 15.1	152.4 ± 16.1
Young adulthood	280.1 ± 15.1	179.0 ± 14.3
Older adulthood	388.3 ± 16.1	205.8 ± 15.1

2.2 Apparatus

2.2.1 ET

The ET consisted of an area of vinyl floor (measuring 120cm x 120cm) enclosed on all four sides by a wooden, painted wall (measuring 50cm in height). A round, opaque plastic box (18 x 14.3 cm) was placed mid-way along a wall of the arena with a single entrance/exit hole (8 x 7 cm) directed towards the centre of the arena. At the beginning of the test, the subject was placed inside the box via a lid. A photograph of the apparatus as viewed from the camera above is shown in **Figure 3.1.**

2.2.2 OF

The open field was identical to the ET enclosure describe above, minus the start box. The floor of the arena was marked into nine areas on the computer software (8 outer and 1 central area) by four lines, each 30cm from one of the walls. At the beginning of each test, the subject was placed into the front left corner of the arena. A photograph of the apparatus as viewed from the camera above is shown in **Figure 3.2**.

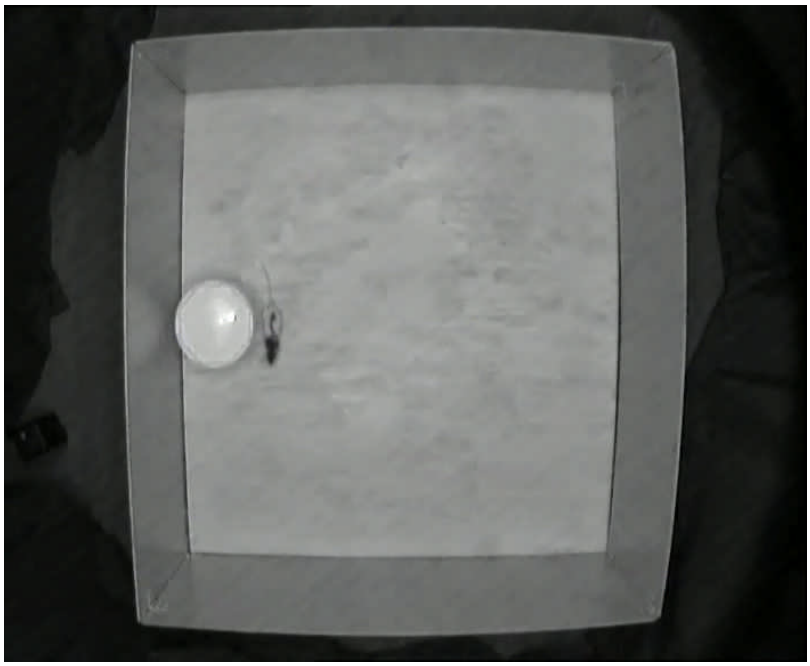

Figure 3.1: photograph of the ET recorded from the overhead camera during this experiment. The rat pictured is parallel to the box's exit.

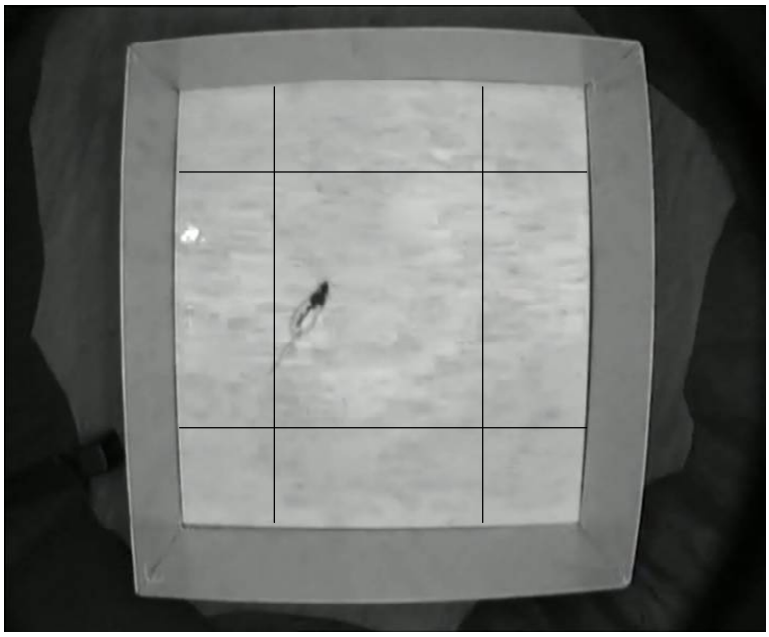

Figure 3.2: photograph of the OF recorded from the camera during this experiment with a rat in the central square. The lines are artificially superimposed on Ethovision XT.

2.2.3 EPM

The EPM consisted of four painted, wooden arms (51 x 11cm) raised 56cm from the ground on a metal frame. Two of the arms had grey wooden walls (closed arms; 40cm high) and the remaining two arms lacked walls (open arms), and the central area was 11cm^2. At the start of the test, the subject was placed into the central area facing a closed arm. A photograph of the apparatus as viewed from the camera above is shown in **Figure 3.3**.

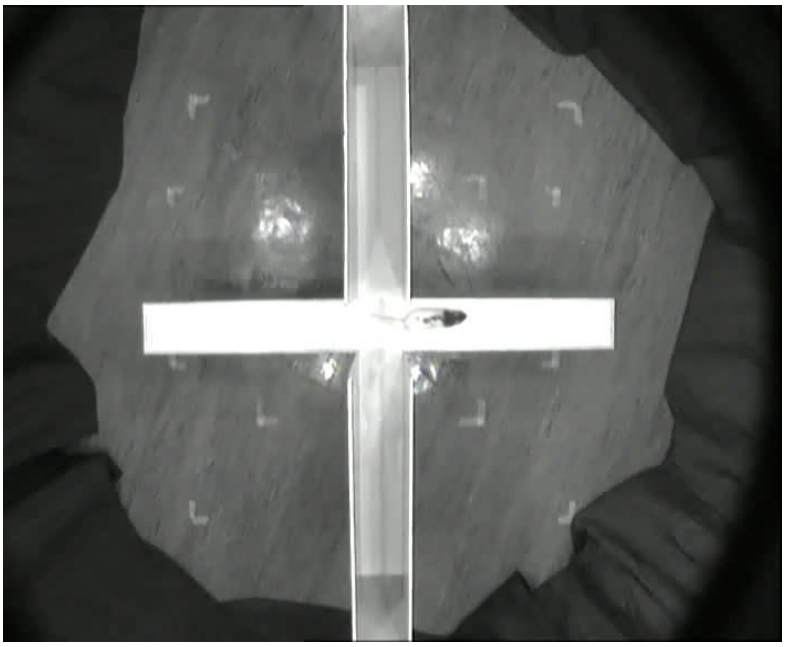

Figure 3.3: photograph of the EPM recorded from the camera during this experiment. The open arms run left to right and the closed arms run top to bottom. The rat pictured has just entered the right open arm.

2.3 Experimental design

The subjects completed the three tests in the following order: ET, OF and EPM. All subjects received the tests in the same order, so that any possible order effects were uniformly distributed across age groups and variance between individuals on each task was minimised. The animals completed one test per day for three consecutive days, and all tests were conducted between

11:00-15:00 hours in the same testing room under dim, white light (approximately 25 lux). The apparatus was surrounded by a black curtain to reduce the number of external cues visible to the subject, and preventing the experimenter from being visible to the subjects. A video camera attached to the ceiling relayed images of each test to a computer. Immediately prior to a test, the subject was transported to the test room in a small, covered box. After each test, the subject was returned directly to the home-cage, and the apparatus was cleaned with a 70% alcohol solution.

Each test lasted 10 minutes, during which the behavioural data were collected either by Ethovision XT software (Noldus Information Technology, The Netherlands) or by manual entry onto a laptop computer running software created within the School of Psychology, University of St Andrews.

2.4 Behavioural Measures

2.4.1 ET

In the emergence test the following behaviours were measured:

a) *enter a new area*: the animal enters a new area (including the start box and sections of the open field as shown in **Figure 3.2**).

b) *peeking from the start box*: an animal puts its head out of the box, at least up to the level of its ears, without exiting the box.

From these measures, *latency to exit to exit the start box* and *total start box re-entries* were recorded.

2.4.2 OF

In the open field, the following behaviours were measured:

a) *enter a new area*: an animal enters a new area of the OF (as shown in **Figure 3.2**) with all four paws.

From this, *total locomotion* (total line crossings) across all areas of the OF were calculated, along with *percentage of time spent in the centre* (total second in the centre, divided by total length of the test in seconds, multiplied by 100). Ethovision was also used to calculate total distance moved (TDM; cms) and the percentage of the total test duration spent mobile.

2.4.3 EPM

In the EPM, the following behaviour patterns were recorded:

a) *enter a new arm or central area*: all four of the animal's paws cross the boundary into a different arm or into the central area.

b) *peek onto an open arm*: the animal puts its head out onto an open arm, at least up to the level of the ears, without entering it.

Total locomotion was defined as the total number of entries into closed and open arms and the central area. *Percentage of entries* into each area were calculated as the number of entries into that area divided by the total locomotion, multiplied by 100. *Percentage of time* spent in a particular area of the apparatus was calculated by dividing the time spent in the area by the total duration of the test, multiplied by 100.

2.5 Statistical Analyses

The data were checked for normality using the Kolmogorov-Smirnov test. Data not normally distributed were subject to a log transformation. Normal and normalized data were analysed using multivariate analyses of variance (MANOVA) with age and sex as between-subject variables and the above behavioural measures as within-subject variables. Variables not normally distributed were analysed using Kruskal-Wallis tests. Planned post-hoc trend tests (polynomial linear/quadratic contrasts) were performed to examine patterns of behavioural change from adolescence to adulthood. Where there was a main effect of age on locomotion measures (TDM and total line crossings in the OF), an analysis of covariance (ANCOVA) was performed using body weight as a covariate. Age x sex interactions are only reported if significant. Pearson's correlation co-efficient tests were used to examine the relationship between measures on the behavioural tests. The data reported in brackets are means ± standard errors (SEMs). Effect sizes are reported as η_p^2.

3. Results

3.1 Emergence test

3.1.1 Latency to Exit the Box

There was a significant main effect of sex ($F_{1, 22} = 5.66$, $p = .026$; $\eta_p^2 = .205$) on latency to exit the box, with females being quicker to do so than males (males: 13.6 ± 2.3 secs; females: 10.0 ± 2.2 secs). There was no main effect of age ($F_{3, 22} = 1.05$, $p = .392$, $\eta_p^2 = .125$).

3.1.2 Total Number of Re-entries

There was a significant effect of age on the total re-entries into the box ($\chi^2_3 = 7.85$, $p = .049$), but no effects of sex ($\chi^2_1 = 0.10$, $p = .919$). Trend tests revealed that a significant linear decrease in cage re-entries with age ($p = .007$; mid-adolescents = 4.3 ± 0.4, late adolescents = 4.7 ± 0.4, young adults = 4.6 ± 0.4, older adults = 3.2 ± 0.4).

3.1.3 Total Peeks from the Box

There was neither a main effect of age ($F_{3, 22} = 2.40$, $p = .096$; $\eta_p^2 = .246$; mid-adolescents: 1.1 ± 0.2; late adolescents: 1.4 ± 0.3; young adults: 0.8 ± 0.2; older adults: 0.5 ± 0.3) nor of sex on the total number of peeks ($F_{1, 22} = 1.22$, $p = .282$; males: 1.1 ± 0.2; females: 0.8 ± 0.2).

3.2 Open Field

3.2.1 Locomotory Measures

For total locomotion, there was a significant main effect of sex ($F_{1, 23} = 8.10$, $p = .009$; $\eta_p^2 = .258$), with females locomoting more than males, and a main effect of age ($F_{3, 23} = 3.51$, $p = .031$; $\eta_p^2 = .307$). Trend analyses revealed a significant linear increase in total locomotion with age ($p = .011$; **Figure 3.4**). After covarying body weight, the main effect of age on total locomotion persisted ($F_{3, 65} = 4.52$, $p = .006$, $\eta_p^2 = .171$).

Total distance moved did not differ between the sexes ($F_{1, 23} = 2.79$, $p = .108$, $\eta_p^2 = .107$), but there was a main effect of age ($F_{3, 23} = 3.39$, $p = .035$, $\eta_p^2 = .301$; **Figure 3.5**). Trend analyses revealed that the TDM increased linearly

across the age groups (p = .011). This main effect of age on TDM persisted after including body weight as a covariate ($F_{3, 65} = 3.04$, p = .035, $\eta_p^2 = .121$).

There was no main effect of age ($F_{3, 23} = 0.23$, p = .874, $\eta_p^2 = .031$; mid-adolescents: 70.4 ±1.7; late adolescents: 74.8 ± 1.7; young adults: 70.3 ± 1.7; older adults: 72.0 ± 1.8) or sex ($F_{1, 23} = 2.94$, p = .100, $\eta_p^2 = .121$; males: 71.6 ± 1.2; females: 73.6 ± 1.2) on the percentage of time spent mobile.

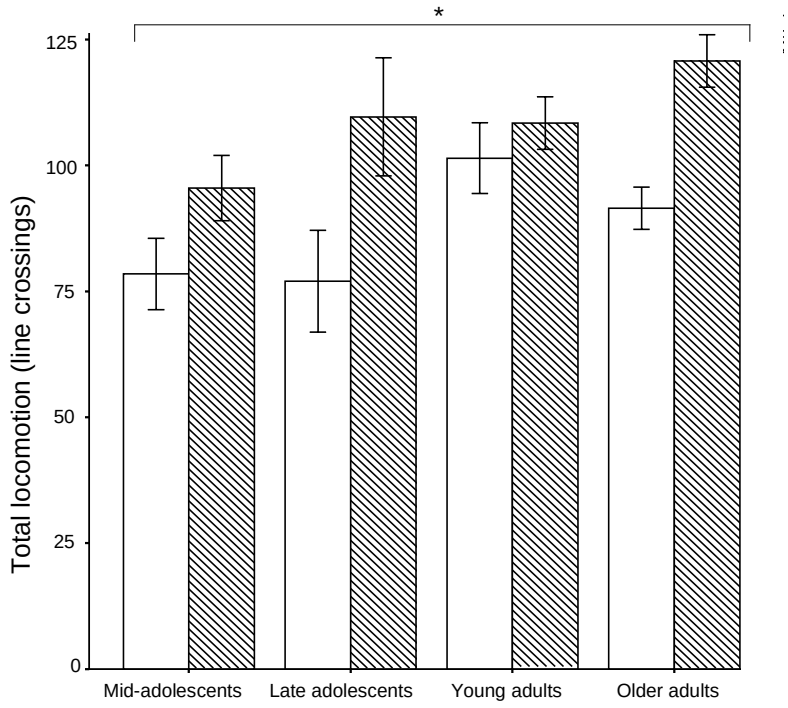

Figure 3.4 Total locomotion in the OF (means ± SEMs). White bars represent males, hatched bars represent females. * indicates a significant main effect of age (p < .05).

3.2.2 Percentage of Time in the Centre

There was no main effect of age ($\chi^2_3 = 6.44$, p = .092; mid-adolescents: 4.2 ± 0.8; late adolescents: 3.6 ± 0.8; young adults: 7.1 ± 0.7; older adults: 5.8 ± 0.8) or of sex ($\chi^2_1 = 0.75$, p = .385; males: 5.2 ± 0.5; females: 5.2 ± 0.5) on the percentage of time spent in the centre of the open field.

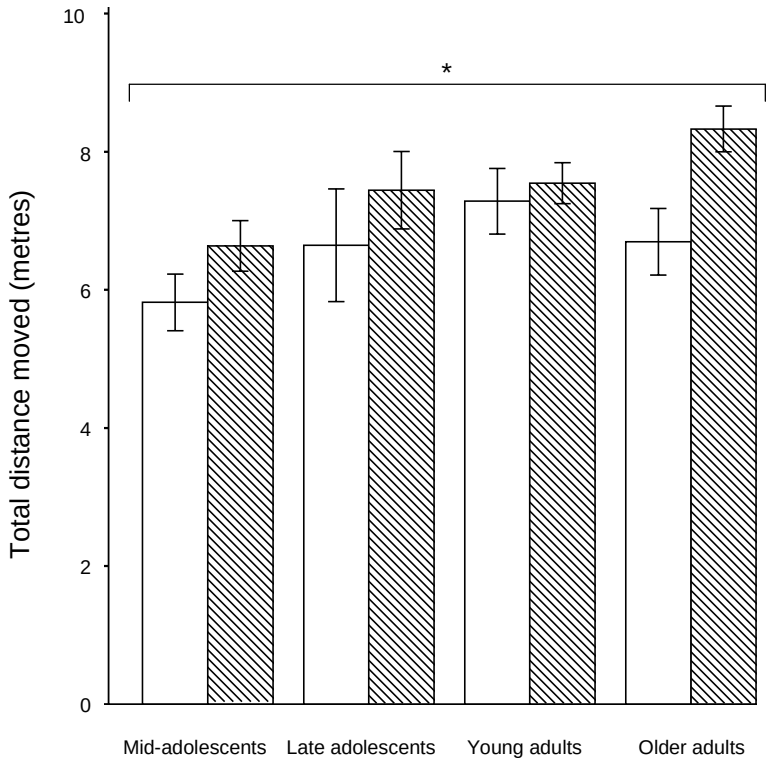

Figure 3.5 Total distance moved (metres) in the OF (means ± SEMs). White bars represent males, hatched bars represent females.* indicates a significant main effect of age ($p < .05$).

3.3 EPM

3.3.1 Total Open and Closed Arm Entries

There was a significant main effect of sex on the total number of open arm entries ($F_{1, 23} = 5.56$, $p = .027$, $\eta_p^2 = 195$), with females making more open arm entries than males (males: 14.9 ± 0.8, females: 17.0 ± 0.7). There was no main effect of age on total open arm entries however ($F_{3, 23} = 2.35$, $p = .099$, $\eta_p^2 = .235$; mid-adolescents: 13.6 ± 1.1; late adolescents: 15.6 ± 1.1; young adults: 17.6 ± 1.1; older adults: 17.4 ± 1.1).

With regards to closed arm entries, there were no main effects of either sex ($F_{1, 23} = 0.26$, $p = .614$, $\eta_p^2 = .011$; males: 18.2 ± 0.7; females: 18.0 ± 0.7) or age ($F_{3, 23} = 1.84$, $p = .168$, $\eta_p^2 = .194$; mid-adolescents: 18.0 ± 1.0; late adolescents: 19.0 ± 1.0; young adults: 18.7 ± 1.0; older adults: 16.5 ± 1.0).

3.3.2 Percentage Open and Closed Arm Entries

There was no main effect of sex on the percentage of open arm entries ($F_{1, 23}$ = 3.18, p = .088, η_p^2 = .122; males: 22.3 ± 0.9; females: 24.2 ± 0.9), although there was a significant main effect of age ($F_{3, 23}$ = 3.09, p = .047, η_p^2 = 287). Trend analyses revealed a linear increase in the percentage of open arm entries with age (p = .046; mid-adolescents: 21.6 ± 1.2; late adolescents: 21.6 ± 1.3; young adults: 24.4 ± 1.2; older adults: 25.7 ± 1.3). This main effect of age persisted when body weight was includes as a covariate ($F_{3, 65}$ = 1.18, p = .339, η_p^2 = .139). There were no significant sex ($F_{1, 23}$ = 2.43, p = .133, η_p^2 = .096; males: 27.6 ± 0.9; females: 25.9 ± 0.9) or age differences ($F_{3, 23}$ = 2.66, p = .072, η_p^2 = .258; mid-adolescents: 28.5 ± 1.3; late adolescents: 28.2 ± 1.3; young adults: 25.5 ± 1.3; older adults: 24.4 ± 1.3) in the percentage on entries into the closed arms of the EPM.

3.3.3 Percentage Time on the Open and Closed Arms

There was a significant main effect of age on the percentage of time on the open arms ($F_{3, 23}$ = 6.56, p =.002, η_p^2 = .461; **Figure 3.6**). Trend analyses revealed that the percentage of open arm time increased linearly across the age groups (p = .001). There was no main effect of sex ($F_{1, 23}$ = 2.25, p =.147, η_p^2 = .089). There were also no sex differences in the percentage of time spent on the closed arms ($F_{1, 23}$ = 1.56, p = .224, η_p^2 = .063), however in corollary with open arm time, there was a main effect of age on time spent on the closed arms ($F_{3, 23}$ = 5.81, p = .004, η_p^2 = .431; **Figure 3.6**), with the percentage duration decreasing linearly across the age groups according to trend analyses (p = .002).

3.3.5 Peeking

There were no significant main effect of sex ($F_{1, 23}$ = 0.25, p = .322, η_p^2 = .130; males: 6.7 ± 0.6; females: 6.2 ± 0.6) or age ($F_{3, 23}$ = 1.14, p =.353, η_p^2 = .130; mid-adolescents: 6.4 ± 0.9; late adolescents: 7.4 ± 0.9; young adults: 5.5 ± 0.9; older adults: 6.5 ± 0.9) on the number of peeks onto the open arms from the centre of the EPM.

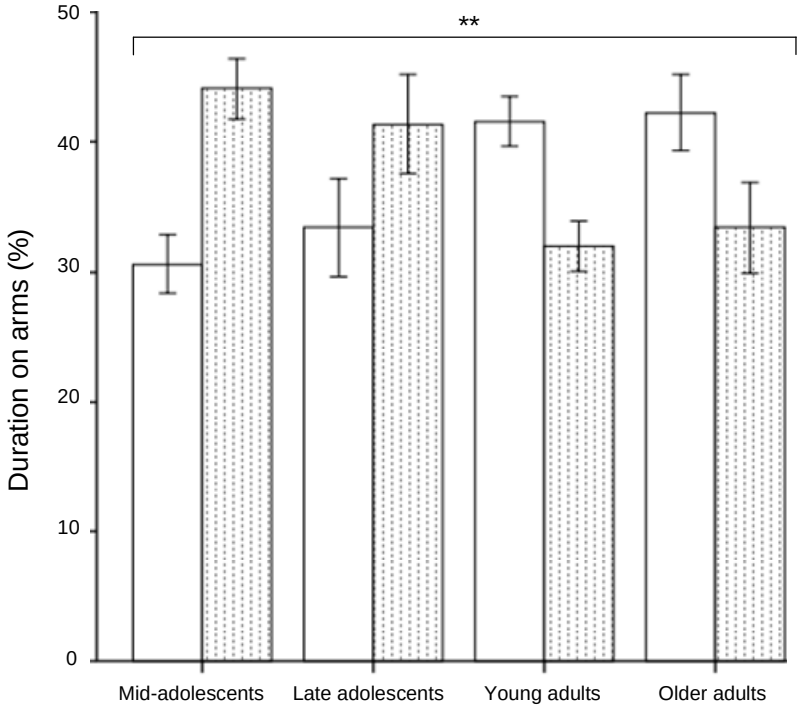

Figure 3.6 Percentage of time spent on open arms (white bars) and closed arms (stippled bars) of the EPM (means ± SEMs). ** indicates a significant main effect of age ($p < .01$).

3.4 Correlations between Performance on the ET, OF and EPM

The percentage of time on the open arms of the EPM was positively correlated with all locomotory measures on the OF: total locomotion ($\rho = 0.292$, $p = .016$), TDM ($\rho = 0.350$, $p = .003$) and the percentage of time spent mobile ($\rho = 0.307$, $p = .011$). The percentage of entries onto the open arms also tended to positively correlate with total locomotion ($\rho = 0.221$, $p = .069$) and TDM ($\rho = 0.222$, $p = .069$) on the OF. The percentage of time spent on the open arms of the EPM negatively correlated with the number of box re-entries on the ET ($\rho = -0.271$, $p = .026$). The percentage of entries onto the open arms of the EPM also negatively correlated with ET box re-entries ($\rho = -0.266$, $p = .028$) and tended to correlate negatively with latency to exit the ET box ($\rho = -0.232$, $p = .057$).

4. Discussion

The aim of this experiment was to examine differences in the behaviour of rats on anxiety-related tests (the ET, OF and EPM) at different stages of adolescence and adulthood. In the ET, animals re-entered the start cage less often across the increasing age groups, and females were faster to first exit the start box than males. In the OF, locomotion (TDM and line crossings) increased across the age groups. In the EPM, there was a linear increase in the percentage of entries onto the open arms and the duration spent there across the age groups, while time on the closed arms decreased. The correlation of measures across the tests support that these pieces of apparatus assess related behaviours, as do the general patterns of greater exploration with age across each test. The results generally support that exploration of novel arenas increases during adolescence and into adulthood. Adolescents are therefore less exploratory in novel environments than adult rats, although this may not necessarily mean that anxiety-like behaviour decreases across the age groups. Locomotion also increases at this time, and this is not explained by the increase in body weight allowing the animal to move further or to be less easily fatigued. Discussed below will be how these results compare to other research, and possible reasons for any inconsistencies.

This experiment replicated many of the findings reported by Lynn and Brown (2009). In the OF, total locomotion increased across age groups in this study, and in the 2009 experiment late adolescents locomoted more than early adolescents. Similarly in both studies, females locomoted more than males, although no interactions with age were found in either experiment. On the EPM, the main effect of age on the percentage time on the open arms was again found, both studies showing an increase in open arm time across the age groups. In addition, this current study also found a significant increase in the percentage of open arm entries with age, an effect that persisted when body weight was considered as a covariate.

In a review on the topic of adolescent behaviour, Spear (2000) reports that the age-related increases in risk-taking related behaviours (including exploration and novelty-seeking) are observed in adolescent rodents, and may aid their transition to an independent adulthood. This study and several

others do not support that 'risk-taking' behaviours are seen in adolescent rats, at least in terms of exploring novel environments. For instance, Doremus, Brunell, Varlinskaya and Spear (2003) found that adolescent rats (pnd 33-35) spent approximately 17% of their time on the open arms of the EPM while adults (pnd 70-75) spent 25% on the open arms, with the same pattern seen for the percentage of open arm entries (25% in adolescents and 38% in adults approximately). These two groups are close in age to the mid-adolescent and young adult rats used in our study, although the overall exploration levels in Doremus and colleagues' experiment may be lower due to the potentially salient differences in pre-test conditions (saline treatment and isolation in the Doremus study, compared to three tests on three consecutive days without pretest manipulations). On the OF, adolescent rats have also been found to by some to be less locomotory compared to adult rats (e.g. Candland & Campbell, 1962).

Other researchers have found that, rather than being less exploratory than adults, adolescents exhibit greater exploration of anxiety-inducing environments in comparison to older rats. For example, Stansfield and Kirstein (2005) found mid-adolescent rats (pnd 35) to locomote more in a circular open field than adults (pnd 60). The fact that a circular open field was used is important, given that rats are thigmotaxic and often prefer corners to long stretches of wall (Treit & Fundytus, 1988). As a circular field contains no corners (the most protected part of the apparatus), adolescents may actually move further in a circular apparatus than a square field as they have no relatively safe corners to remain in, rather than because they are less 'anxious'. Stansfield and Kirstein also did not include female rats in their experiment and only used two age groups, including the youngest possible age that a rat is classified as an adult (pnd 60) rather than an older adult group.

Another study (Philpot & Wecker, 2008) found that mid-adolescent rats (pnd 31) moved a greater total distance in a square open field than late adolescent animals (pnd 56), using apparatus that was scaled to the crown-to-rump length of both age groups. This is in direct contrast to our finding that younger animals locomote less (in terms of line crossings and TDM) than adult animals in the OF, even when body weight is statistically controlled for.

What remains debatable however is whether an apparatus that is scaled to the crown-to-rump length of a young rat is actually perceived in the same way as the full-sized apparatus is to an adult rat. In other words, perception of an apparatus and the risks involved in its exploration may not scale. Scaling is also perhaps less natural, in that adolescent rats do not explore a reduced size environment; rather, adolescents access the same burrow systems and over-ground trails as adult rats do (Calhoun, 1963). This methodological difference may thus contribute to the contrasting findings of these two studies.

While the main effects of age in Lynn and Brown's experiment (2009) largely echo those reported in this chapter, what is noticeable is the lack of sex differences in open arm behaviour in the current study. Lynn and Brown (2009) found that on the EPM, females generally locomoted more and spent a greater percentage of their time on the open arms than males, while this chapter reports no sex differences in EPM open arm activity, only in OF locomotion and ET box exit latencies and re-entries. Sex differences are however reported robustly reported across the literature in adults at least, and especially on the EPM (e.g. Aguilar et al., 2003; Chapter 2; Johnston & File, 1991; Zimmerberg & Farley, 1993). EPM behaviours may thus be more susceptible to methodological variations, and one possible explanation for these inconsistencies in sex differences across the two studies is the difference in testing schedule. Lynn and Brown's (2009) experiment only used two tests, and the subjects had one week between performing the OF and the EPM. In the current experiment, not only did the rats complete three tests, but they also did so over three consecutive days. Research has shown that behaviour on the EPM is sensitive to the prior conditions the rats have experienced: these range from social isolation to prior exposure to the EPM itself (e.g. Doremus-Fitzwater, Varlinskaya & Spear, 2009; Griebel, Belzung, Misslin & Vogel, 1993; Rodgers, Lee & Shepherd, 1992). However, the effects of prior testing in other pieces of behavioural apparatus on subsequent EPM performance have received relatively little attention (Doremus-Fitzwater & Spear, 2007; Lister, 1987; Rodgers and Cole, 1993), despite the fact that using a battery of tests is a common approach in behavioural pharmacology. Even less is known about how adolescents respond to such manipulations, although one study has reported age differences in EPM behaviour following

different pre-test manipulations (Doremus-Fitzwater et al., 2009). More research onto the effects of pre-test manipulations, including the running of multiple tests, on sex and age differences in anxiety-related behaviour is therefore warranted.

There are several strengths to the design of the current experiment. Firstly, there is a good sample size, which includes both sexes (minimum of 9) for each age group. In addition, the use of animals across four age groups, rather than comparing one set of adolescents to one set of adults meant that patterns of behaviour across adolescence and adulthood could be examined, rather than merely making pair-wise comparisons. By doing this, the blur of mixing, for example, early and mid-adolescents, together into one adolescent group was also avoided. The use of multiple tests and the correlation of their measures helps to support the reliability of these results, rather than extrapolating about the ontogeny of these behaviours from the use of a single test, as can often be seen in the literature. The correlations support that the tests measure related behaviours, as do the general patterns of greater exploration with age.

In summary, this experiment has shown that exploration of novel pieces of apparatus related to anxiety-like behaviour increases with age, with adolescents therefore showing lower levels of exploration than adults (differences which are not explained by the variation in body weight alone). This is supported by other research, and by the strong design of the study, with contrasting findings potentially resulting from salient methodological differences. Questions remain however as to how the different sexes and age groups respond to manipulations occurring before tests of anxiety-related behaviour, including the order of testing. This information is essential to gather, given that the use of several tests is beneficial in contributing to the richness of data in such experiments. This issue is directly addressed in Chapter 4.

Chapter 4: The Effects of Testing Order on Elevated Plus-Maze and Locomotor Box Behaviour in Adolescent and Adult Male and Female Rats

1. Introduction

As highlighted in the previous chapter discussion, little research has focused on the effects of repeat testing on anxiety-like behaviour in adult and adolescent male and female rats. Such research is important given the use of test batteries by behavioural pharmacologists in preclinical research, and because by their nature, anxiety-like behaviour as measured by tests such as the elevated plus-maze be particularly susceptible to prior manipulations and exposures to other novel environments (as will be discussed in more detail below). The aim of this experiment was to examine if adolescent and adult male and female rats responded differently on the common test of anxiety-like behaviour after completing a different test, not assessing anxiety itself (the locomotor box. This was achieved by comparing their behaviour to animals that performed the EPM before the LB test.). As locomotor activity has been considered as a confounding variable in EPM performance, a secondary aim was to examine EPM behaviour while controlling for independent measure of ambulation – beam breaks in the locomotor box.

Running multiple tests to assess changes in anxiety-like behaviour (as per Chapter 3) provides researchers with a rich data set of behavioural shifts and changes, and allows for the tests' ability to examine the same or similar behaviours to be assessed by running correlations between different tests' measures. However, the potential downside of using a battery of tests is that running multiple tests, and other pre-test experiences such as social isolation, saline injection or handling, may affect the behaviour of the animals. This becomes especially important when comparing groups of animals, as the behaviour of rats of different ages and sexes may actually be affected differently to one another. Behaviour on the EPM has already been shown to be susceptible to a variety of pre-test manipulations. Previous studies have examined the effects of other tests on EPM behaviour, beginning with multiple exposures to the EPM itself. Such studies have generally reported that

rodents spend less time on the open arms on their second exposure (e.g. Dawson, Crawford, Stanhope, Iversen & Tricklebank, 1994; Espejo, 1997; File, Zangrossi, Viana & Graeff, 1993; Griebel, Belzung, Misslin & Vogel, 1993; Rodgers et al., 1992). The fact that prior behavioural testing influences subsequent EPM performance has implications for test batteries, in which rodents are exposed to a number of behavioural tests in succession and where test order might confound results (Tecott & Nestler, 2004). However, the effects of prior testing in other pieces of behavioural apparatus on subsequent EPM performance have received relatively little attention (Doremus-Fitzwater & Spear, 2007; Lister, 1987; Rodgers & Cole, 1993).

Surprisingly little data has been collected on sex differences in the effects of procedural and methodological variables on EPM behaviour (Doremus-Fitzwater et al., 2009), despite the robust sex differences reported in general EPM performance (Johnston & File, 1991). Pre-test manipulations, such as exposure to novel environments, may affect the performance of male and female rats differently, and such manipulations have been argued to decrease the extent of the sex difference in EPM performance (Doremus-Fitzwater et al., 2009; Lynn and Brown, 2010).

Few studies have investigated the effects of prior behavioural testing on the EPM performance of rodents of different ages. As some other researchers have found (e.g. Candland & Campbell, 1962; Doremus et al., 2003), this thesis has reported that adolescent rats are typically less exploratory than adult rats in tests of anxiety-like behaviour (Chapter 3). Only one study has systematically investigated the effects of exposure to a novel environment on EPM in adolescent and adult rats (Doremus-Fitzwater et al., 2009). This study reported that either a) exposure to a novel environment prior to testing (moderate manipulation) or b) a saline injection and rehousing followed by exposure to a novel environment (large manipulation) increased open arm activity in adolescents rats compared to animals tested directly from the home cage, while only the large manipulation increased open arm activity in adults relative to controls. Adolescent and adult rats therefore appear to differ in their response to pre-test manipulations, and the direction of age differences in EPM performance is likely to be influenced by the nature of pre-

test events. When comparing male and female rats, as the manipulations increased in magnitude, the sex differences in behaviour decreased. This fits with the fact that in the previous chapter that anticipated sex differences were not found on the EPM – the final of three tests over three consecutive days.

In this study, the effects of exposure to a small, novel environment (the LB) immediately prior to testing on the EPM were examined in adolescent and adult male and female rats. The EPM was used given the previous literature suggesting its sensitivity to pre-test manipulations and methodologies, along with the fact that in the previous chapter, sex differences may not have been seen on this test precisely because of the use of multiple tests. The LB was chosen as it is not a test of anxiety-like behaviour itself; it provides an independent measure of ambulation, as well as the fact that this apparatus is commonly used in drug studies and in pharmacological test batteries. In addition, given that locomotor activity has been suggested to be a confounding variable in analyses of EPM performance (Dawson and Tricklebank, 1995; Dawson et al., 1995) and has been found to load on the same component as open arm activity in factor analyses (Cruz et al., 1994; Doremus et al., 2006), the locomotor activity box also provided an independent measure of locomotion that could be used as a co-variate in analyses of the EPM data. We hypothesised that prior exposure to the novel apparatus would influence subsequent performance on the EPM (and *vice versa*), and that the effect could differ between the adult and adolescent rats, and between the sexes.

2.0 Methods

2.1 Subjects and Housing

The subjects were 40 mid-adolescent (pnd 39-41; 20 male, 20 female) and 32 adult rats (pnd 211-217; 16 male, 16 female) Lister Hooded rats, bred in-house from stock supplied by Harlan, U.K. All animals were weaned at pnd 21 and housed in pairs with same-sex littermates in wire-topped cages (measuring 25cm x 45cm x 15cm) with *ad libitum* access to soy-free rodent pellets and water. Housing rooms were controlled for temperature and humidity, and maintained on a 12-hour light: dark cycle (lights on 7am; light

level of 90 lux). Animals were weighed weekly until pnd 60, and monthly thereafter. At the beginning of testing adult females weighed an average of 235g and adult males weighed an average of 426 g. Adolescent females weighed an average of 77g and adolescents males weighed an average of 86g. All appropriate guidelines and requirements were adhered to, as set out in the *Principles of Laboratory Animal Care* (NIH, Publication No. 86-23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986.

2.2 Apparatus

2.2.1 EPM

The elevated plus-maze consisted of four painted, wooden arms (51cm long x 11cm wide) that were raised 56cm from the ground on a metal frame. Two of the arms had 40cm high wooden walls (closed arms) and the remaining two arms lacked walls (open arms), and the central area was 11cm^2. The apparatus was painted grey and located in a testing room with dim white lighting (approximately 25 lux).

2.2.2 LB

The locomotor box consisted of a transparent, perspex box (46cm long x 24cm wide x 22cm high) which was surrounded by a set of photo-beams. Breaks in the photo-beams were automatically recorded by a computer running MotorMonitor software (version 4.14, Hamilton-Kinder, L.L.C., U.S.A.). The set of six locomotor boxes were in a small testing room, separate from the EPM testing room.

2.3 Experimental design

Half of the subjects performed the LB test first (10 adolescent males, 10 adolescent females, 8 adult males, 8 adult females) and half performed the EPM test first. Each subject was therefore tested only once in the EPM (10-minute test) and once in a locomotor box (10-minute test). The animals completed both tests consecutively without a break. Animals were carried to

and from the testing rooms via a carry box with a solid lid (45 x 28 x 13 cm). All tests were carried out between 14:00 – 17:30.

At the start of the EPM test, the subject was placed into the central area, facing a closed arm and left for 10 minutes. Behavioural data were recorded directly by the observer onto a laptop computer running in-house data collection software. After each test, the apparatus was cleaned with a 70% alcohol solution and allowed to dry. For the LB test, the animal was placed in a locomotor box, with the test beginning once the lid was placed on top. The experimenter left the room for the duration of the test, returning as soon as the 10 minutes were complete.

2.4 Behavioural Measures

2.4.1 EPM

In EPM tests, the following behaviour patterns were recorded:

(i) *Enter a new arm or central area*: all four of the animal's paws cross the boundary into a different arm or into the central area.

(ii) *Peek onto an open arm*: the animal puts its head out onto an open arm at least up to the level of the ears without entering it.

From these measures, the computer programme calculated total frequencies of behaviour patterns, as well as the total durations of time spent in each area of the apparatus (open arms, closed arms, central area). Total locomotion was defined as the total number of entries into closed arms, open arms and the central area. Percentage of entries onto the open arms was calculated by dividing the number of open arm entries by total locomotion and multiplying by 100. Percentage of time spent on the open arms was calculated by dividing time on the open arms by total test length and multiplying by 100.

2.4.2 LB

In the LB, the following activities were recorded:

a) *basic movements*: an animal's body breaks a photobeam, while another simultaneously reforms i.e. these are the whole body movements.

b) *fine movements*: an animal's body breaks a photobeam *without* another becoming reforming; these movements may include rearing, sniffing and grooming.

From these measures, the total number of basic movements and fine movements over the five minute test period were calculated.

2.5 Statistical Analyses

The data were checked for normality using the Schapiro–Wilk test, and non-normally distributed data were log transformed. Normal and normalised data were analysed using multivariate analyses of variance (MANOVA), with sex, age and order of testing as between-subject variables and the behavioural measures as within-subjects measures. An ANCOVA was also performed, with locomotor box basic ambulation as the co-variate, Separate MANOVAs were conducted on the EPM and LB data. Where significant main effects of age were detected, an ANCOVA was run including age as the between-subject variable, body weight as the co-variate, and the measures showing a main effect of age as the within-subjects variables. The Kruskal-Wallis test was used to analyse non-normally distributed variables. Data reported in brackets are means ± SEMs. Effect sizes are reported as η_p^2. Non-significant interactions are not reported.

3. Results

3.1 EPM

3.1.1 Total Open and Closed Arm Entries

There were no effects of age ($F_{1, 64} = 0.17$, $p = .686$, $\eta_p^2 = .003$; adolescents: 12.6 ± 0.7; adults: 12.2 ± 0.8) on total open arm entries, although females did enter open arms significantly more than males ($F_{1, 64} = 6.91$, $p = .011$, $\eta_p^2 = .097$; males: 11 ± 0.7; females: 13.7 ± 0.7). This sex difference persisted when locomotor box ambulation was taken into account ($F_{1, 64} = 4.18$, $p = .046$, $\eta_p^2 = .073$). There was no effect of order ($F_{1, 64} = 2.71$, $p = .105$, $\eta_p^2 = .041$; LB-EPM: 13.2 ± 0.7; EPM-LB: 11.5 ± 0.7), and no interactions between sex, age and testing order.

For total closed arm entries on the EPM, there were no significant effects of sex ($F_{1, 64}$ = 0.66, p = .420, η_p^2 = .010; males: 16.5 ± 0.7; females: 15.7 ± 0.7) or age ($F_{1, 64}$ = 0.98, p = .327, η_p^2 = .015; adolescents: 15.7 ± 0.6; adults: 16.6 ± 0.7). However, animals that completed the LB test first made significantly fewer closed arm entries on the EPM than animals that completed the EPM first ($F_{1, 64}$ = 7.41, p = .008, η_p^2 = .104; LB-EPM: 14.9 ± 0.7; EPM-LB: 17.4 ± 0.7). This effect of order persisted after locomotor box ambulation was statistically accounted for ($F_{1, 64}$ = 8.72, p = .005, η_p^2 = .141).

3.1.2 Percentage Open and Closed Arm Entries

There was a significant main effect of sex on the percentage of open arm entries ($F_{1, 64}$ = 6.41, p = .014, η_p^2 = .091), with females making a higher proportion of entries onto the open arms than males (**Figure 4.1**). This main effect of sex persisted when locomotor box ambulation was added as a co-variate ($F_{1, 64}$ = 6.34, p = .015, η_p^2 = .107). A main effect was also found for order of testing ($F_{1, 64}$ = 6.86, p = .011, η_p^2 = .097), with animals that performed the LB test first making a higher proportion of open arm entries than those performing the EPM first (**Figure 4.1**). This main effect persisted when ambulation in the locomotor box was controlled for ($F_{1, 64}$ = 5.44, p = .022, η_p^2 = .096). There was no main effect of age ($F_{1, 64}$ = 1.01, p =.318, η_p^2 = .016; adults: 22.1 ± 0.9 %; adults: 20.8 ± 1.0 %).

As a corollary to open arm entries, males made a higher percentage of closed arm entries than females ($F_{1, 64}$ = 4.91, p = .030, η_p^2 = .071; males: 45.5 ± 2.6%; females: 42.0 ± 2.6%). This main effect of sex persisted when ambulations in the LB were added as a covariate ($F_{1, 64}$ = 5.44, p = .022, η_p^2 = .096). Animals that performed the LB first made a lower proportion of closed arm entries than did animals that performed the EPM first ($F_{1, 64}$ = 8.13, p = .006, η_p^2 = .113; LB-EPM: 26.7 ± 1.0%, EPM-LB: 30.4 ± 1.0%). This main effect or order persisted when LB ambulation was statistically controlled for ($F_{1, 64}$ = 5.61, p = .022, η_p^2 = .096). There was no main effect of age ($F_{1, 64}$ = 0.68, p = .414, η_p^2 = .010; adolescents: 28.0 ± 0.9 %; adults: 29.1 ± 0.1 %).

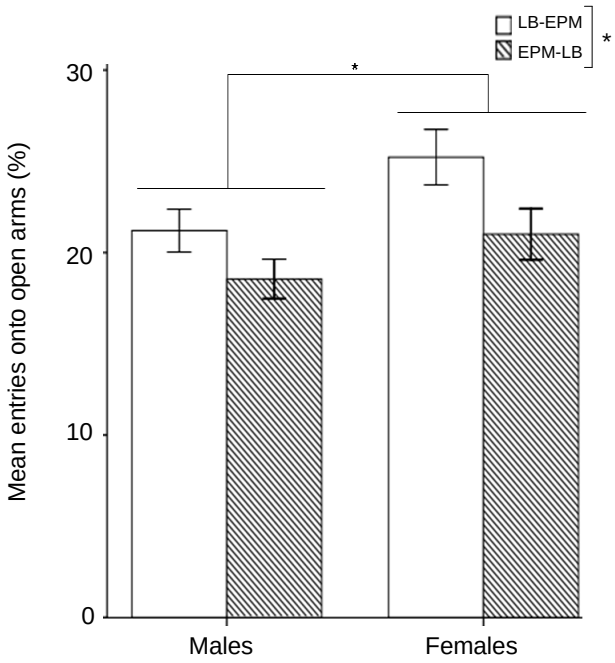

Figure 4.1 Percentage of entries onto the open arms of the EPM for both sexes and orders of testing (means ± SEMs). * indicates a significant main effect at $p < .05$.

3.1.3 Percentage Open and Closed Arm Durations

There was a main effect of sex on the percentage of time spent on the open arms ($F_{1, 64} = 6.22$, $p = .015$, $\eta_p^2 = .089$), with females spending more time there than males (**Figure 4.2**). This difference persisted when LB ambulation was include as a covariate in the analyses ($F_{1, 64} = 4.56$, $p = .037$, $\eta_p^2 = .079$) .There was also an effect of order ($F_{1, 64} = 5.51$, $p = .022$, $\eta_p^2 = .079$), with animals that performed the LB test first spending more time on the open arms than animals that performed the EPM first (**Figure 4.2**). This main effect of order was no longer significant once LB ambulation was controlled for ($F_{1, 64} = 2.77$, $p = .102$, $\eta_p^2 = .050$).There was no main effect of age ($F_{1, 64} = 0.41$, $p = .524$, $\eta_p^2 = .006$; adolescents: 29.6 ± 2.1 %; adults: 31.6 ± 2.3 %).

There were no significant sex ($F_{1, 64} = 0.65$, $p = .425$, $\eta_p^2 = .010$; males: 45.0 ± 2.5 %; females: 42.1 ± 2.5 %) or age differences ($F_{1, 64} = 2.93$, $p = .092$, $\eta_p^2 = .044$; adolescents: 46.6 ± 2.4 %; adults: 40.6 ± 2.6 %) in the percentage of time spent on the closed arms of the EPM. However, animals that performed the EPM first spent more time on the closed arms than did

animals that performed the LB first ($F_{1, 64}$ = 6.50, p = .013, η_p^2 = .092; LB-EPM: 39.4 ± 2.6%, EPM-LB: 48.1 ± 2.6%). This main effect was reduced to a tendency once LB ambulation was statistically controlled for ($F_{1, 64}$ = 3.35, p = .079, η_p^2 = .059).

3.1.4 Peeking

Male rats peeked onto the open arms of the EPM significantly more often than females (χ^2_1 = 5.39, p = .020; males: 9.7 ± 0.8, females: 6.3 ± 0.8). There were no main effects of order (χ^2_1 = 0.25, p = .614; LB-EPM: 8.0 ± 0.7; EPM-LB: 8.0 ± 0.7) or age (χ^2_1 = 2.31, p = .128; adolescents: 6.9 ± 0.6; adults: 9.0 ± 0.7) on the total number of peeks.

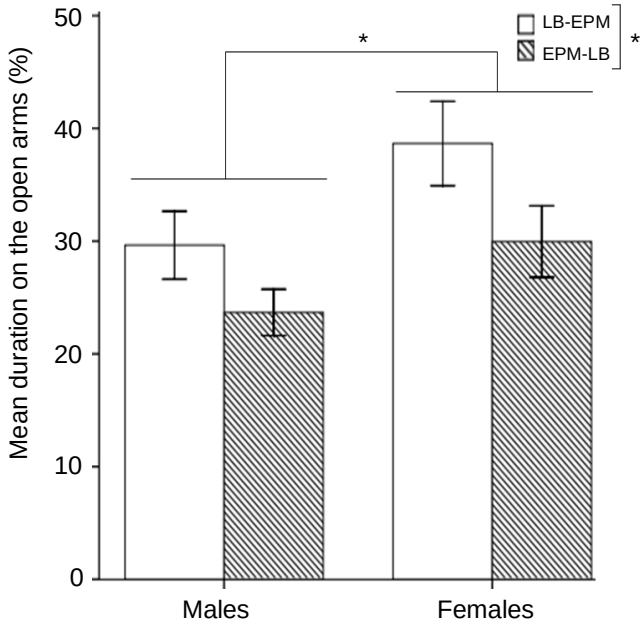

Figure 4.2 Percentage of time spent on the open arms of the EPM (means ± SEMs). * indicates a significant main effect at p < .05.

3.2 Locomotor Box

Females made more whole body movements in the LB than males ($F_{1, 61}$ = 16.41, p < .001, η_p^2 = .212; **Figure 4.3**). Animals that performed the LB first also made more beam breaks than animals that completed the EPM first ($F_{1,}$

$_{61}$ = 4.67, p = .035, η_p^2 = .071; **Figure 4.3**). There was also an effect of age, with adolescents breaking more beams than adults ($F_{1, 61}$ = 14.90, < .001, η_p^2 = .196; adolescents: 1283.5 ± 34.6 beam breaks per test, adults: 1084.2 ± 38.3 beam breaks per test). This main effect of age was reduced to a tendency when body weight was included as a co-variate ($F_{1, 66}$ = 3.60, p = .062, η_p^2 = .052).

Females also made significantly more fine body movements in the LB than male rats ($F_{1, 61}$ = 7.90, p = .007, η_p^2 = .115; males: 583.4 ± 18.8; females: 657.5 ± 18.4). There was also a main effect of age, with adults making more fine movements than adolescents ($F_{1, 61}$ = 8.20, p = .006, η_p^2 = .119; adolescents: 582.7 ± 17.6; adults: 658.2 ± 19.5). This significant age difference on fine movements persisted when body weight was statistically controlled for ($F_{1, 66}$ = 12.77, p = .001, η_p^2 = .162) There was also a tendency for animals that performed the LB first to make more fine movements than rats that completed the EPM first ($F_{1, 61}$ = 3.74, p = .058, η_p^2 = .058; LB-EPM: 645.9 ± 18.7; EPM-LB: 595.0 ± 18.6).

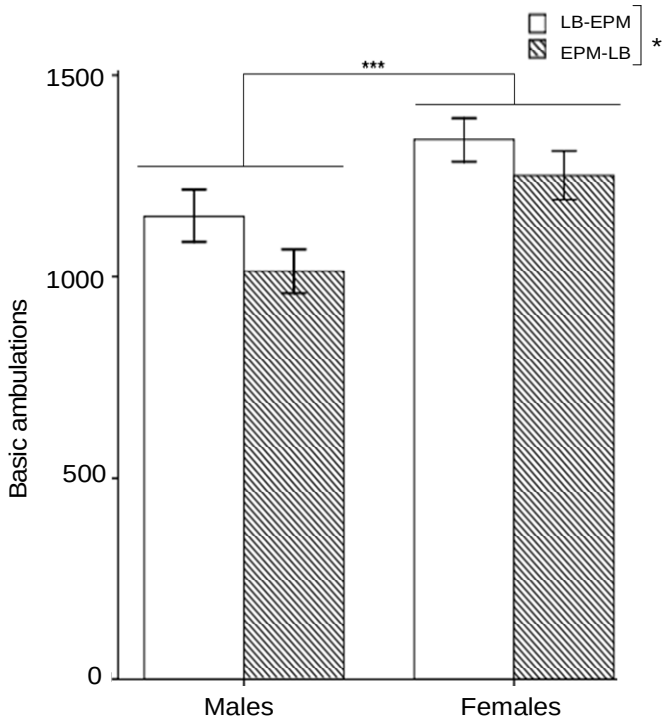

Figure 4.3 Basic movements made in the LB (means ± SEMs). *** indicates a significant main effect at p < .001, * indicates a significant main effect at p < .05.

4. Discussion

The aims of this experiment were a) to examine the effects of testing order on the behaviour of adult and adolescent male and female rats when exposed to the EPM and to the LB and b) to examine whether differences in EPM behaviour could be explained in terms of locomotion. In terms of order effects, LB-first animals made a greater percentage of open arm entries and spent more time on the open arms than rats that completed the EPM first. In corollary of this, rats that completed the EPM before the LB made more closed arm entries (total and percent) and spent more time on the closed arms relative to animals that completed the LB test first. In the locomotor box, rats that completed the LB first broke more beams than EPM-first animals, and tended to make more fine body movements. In terms of sex differences, females made more open arm entries (total and percentage) than males. The percentage of time spent on the open arms followed the same pattern as open arm entries, with females spending a greater percentage of time on the open arms than males. In corollary to this, males made a greater proportion of closed arm entries than females, and also peeked onto the open arms more than female rats. These sex differences in EPM behaviour persisted when an independent measure of locomotion, LB ambulation, was statistically controlled for. In the locomotor box, females made both more basic and fine body movements than male rats. With regards to age, adult and adolescent performance on the EPM did not significantly differ. In the LB, adolescents broke more beams than did adults, although this difference was reduced to a tendency once body weight was taken into account.

Overall, these results suggest that prior testing in a novel apparatus increased open arm exploration in the EPM, in line with studies showing that other moderate manipulations increase open arm activity (e.g. Andrews & File, 1993; Santucci, Daud, Almeida & de Oliveira, 1994). This increase is not explained by manipulations in general levels of locomotion. In contrast, manipulations that are potentially more stressful to the animal reportedly decrease open arm activity (e.g. Adamec, Sayin & Brown, 1991; Da Silva, Ferreira, Carobrez. & Morato, 1996). Our study supports the argument that the effect of prior events on EPM performance varies with the nature of the manipulation (Hogg, 1996).

Relatively few studies have investigated the effects of prior testing in novel behavioural apparatus on subsequent EPM performance without additional manipulations, such as injections, also being performed. Our finding that prior exposure to a novel environment increased open arm activity in the EPM is in agreement with the work of other researchers on the topic. For example, prior exposure to a small novel arena (Rodgers & Cole, 1993) or hole-board apparatus (Lister, 1987) increases subsequent open arm activity in the EPM in mice. Similarly, Doremus-Fitzwater and colleagues (2009) found that exposure of rats to a novel context for 30 minutes prior to the EPM increased the proportion of open arm entries. Doremus-Fitzwater and colleagues suggested that decreased anxiety-like responses may be due in part to a general locomotor increase in the second test, with more active animals being more likely to sample the open arms of the apparatus. Their results provided some evidence that pre-test manipulations lead to greater locomotion in the EPM, as indexed by increased total closed arm entries (Doremus-Fitzwater et al., 2009). In contrast, our study found no effect of previous LB testing on total arm entries in the EPM, and, in fact, prior LB testing decreased total closed arm entries. This difference in results could potentially be explained by the shorter length of exposure to a novel environment prior to EPM testing in our study (10 minutes) compared to the previous study (30 minutes). However, our study also found that prior EPM testing subsequently suppressed locomotor activity in the LB, indicating that prior behavioural testing can lead to a decrease in locomotion. Therefore, the effects of previous exposure to a novel environment on subsequent EPM performance are not readily explained in terms of changes in locomotor activity.

The fact that prior behavioural testing increases the proportion of open arm activity may initially appear at odds with previous research demonstrating that a second exposure to the EPM itself is associated with increased anxiety-like behaviour, i.e. a decrease in the amount of time on the open arms and/or proportion of entries onto the open arms (e.g. Dawson et al., 1994; Espejo, 1997; File et al., 1993; Griebel et al., 1993; Rodgers et al., 1992). The results of our study support the suggestion that the type of behavioural task previously encountered can influence the effects on subsequent behavioural

testing (Brown & Nemes, 2008). Exposure to an enclosed novel environment (such as a locomotor box, hole-board apparatus or small novel arena) can lead to rodents spending more time on the open arms (e.g. Rodgers & Cole, 1993). In contrast, exposure to the EPM (which possibly induces greater stress as it is reportedly a test of anxiety-like behaviour) is known to suppress open arm activity in the subsequent tests on the EPM (e.g. Dawson et al., 1994) and also locomotor activity in a novel, enclosed environment (this study). In test batteries, the effects of prior behavioural testing on subsequent behavioural performance will therefore depend upon the types of apparatus used.

In terms of sex differences, this study found that female rats made a greater percentage of entries onto the open arms and spent more time on these arms than males. These results are in line with previous studies of rats (e.g. Aguilar et al., 2003; Chapter 2; Johnston & File, 1991; Leussis & Andersen, 2008). As a corollary, males made a greater proportion of closed arm entries, were slower to enter an open arm and peeked onto open arms more often than females. Females also exhibited higher levels of locomotion than males in the LB, as shown in previous studies (Aguilar et al., 2003; Engeland, Kavaliers & Ossenkopp, 2003), although differences in locomotion did not eliminate these sex differences in EPM behaviour. While sex differences in EPM performance have been interpreted as showing that female rats are less anxious, or more fearful, than males (Aguilar et al., 2003; Leussis and Anderson, 2008; Zimmerberg & Farley, 1993), Johnson and File (1991) cautioned that conclusions about sex differences in anxiety cannot easily be drawn from sex differences in open arm activity, as the EPM might not measure the same variables in male and female rats (also see Fernandes et al., 1999).

The lack of an interaction between sex and order of testing indicates that the sex differences in behaviour were not diminished by pretesting experience, in contrast to previous studies (Doremus-Fitzwater et al., 2009; Chapter 3 of this thesis). Doremus-Fitzwater and colleagues (2009) reported a sex differences in EPM performance in rats when the amount of pre-test manipulation was low or moderate, but a lack of sex difference following large pre-test manipulation, when the percentage of time spent on the open arms

by males rose to levels equivalent to those reported for females. Similarly, the previous chapter of this thesis reported a lack of sex differences in EPM performance in adult rats that had undergone behavioural testing on the two days preceding EPM testing. Both of these studies used larger pre-test manipulations than that used in the current study. In the current experiment, a relatively short exposure to a locomotor box did not influence the direction or extent of the sex differences in open arm and closed arm activity. Therefore, the sex differences in EPM performance is seemingly relatively robust, except following large pre-test manipulations, when male performance becomes aligned with that of females (e.g. Doremus-Fitzwater et al., 2009).

In our study, adolescents and adults did not differ in their behavioural responses on the EPM, and order of testing did not have differential effects on adolescents and adults. The only significant effect of age was on locomotor activity in the LB, with adolescents breaking more beams than adults: an effect that disappeared once body weight was controlled for. In contrast, Doremus-Fitzwater and colleagues (2009) reported that the response to pre-test manipulation did differ with age: moderate or large pre-test manipulations increased open arm activity in adolescents, while only large manipulations increased open arm activity in adults. In the current study, a relatively small pre-test manipulation increased open arm activity in both adolescents and adults. Comparing the exploration levels between mid-adolescents (pnd 34-39) and adolescents (pnd 39-41) across the two chapters, and between older adults (pnd 104-109) and adults (pnd 211-217), adults in the current experiment appear to be exhibiting lower open arm activity compared to older adults in Chapter 3, while adolescents in both studies show similar levels. For instance, while older adult rats spent 44% of their time on the open arms in the previous experiment, adults in the current study spent only 32 % of their time there. Mid-adolescents on the other hand spent 32% of their time on the open arms of the previous experiment, while adolescents in the current study spent 30% of their time there. Therefore, it appears as if adolescent open arm activity was relatively unaffected by the order of testing, while adult rats' level of exploration decreased to adolescent-like levels.

Methodological differences may go some way to explaining the inconsistency in age differences between these two chapters and compared

to Doremus and colleagues' work. Comparing chapters, while the tests were run immediately after one another in this study, the EPM was the final of three behavioural tests, ran one a day over three consecutive days, with research already showing that type of pre-test manipulation is important (Doremus-Fitzwater et al., 2009). In the study by Doremus-Fitzwater and colleagues (2009), the percentage of time on the open arms of the EPM for subjects tested directly from their home-cages was much lower than for our subjects who were tested on the EPM first. This could result from strain differences or other procedural factors. If subjects are starting from a lower baseline level of open arm activity, they might be more resistant to pre-test manipulations, and this resistance could manifest differently in adults and adolescents. The type of pre-test manipulation and baseline levels of EPM performance, therefore, are both potentially important factors in determining how animals will respond to events that occur prior to behavioural testing.

In summary, this study has shown that prior exposure to a novel environment increases open arm activity on the EPM in both male and female adult and adolescent rats; effects that are not explained by alterations in locomotion levels alone. Along with the large body of research on factors than can effect EPM performance (including handling, altered housing conditions, isolation and so on; see Hogg, 1996 for a review), this should serve as a warning for researchers using batteries of tests, that are often used in pharmacological research with little or no regard as to the effect of testing order (Tecott & Nestler, 2004). Better characterisation and understanding of how one test may affect an animal's behaviour on another is needed. There is scope for using such findings to benefit research protocols, as there may be ways to exploit the effects of previous test experience on the EPM to create conditions more suited for testing the variable of interest, for example, by increasing baseline open arm behaviour before examining the potential anxiogenic effects of a drug. Further research, including studies of the response of the stress hormone corticosterone, may help elucidate how novel environment exposure and other pre-test manipulations affect behaviour on the EPM and how this compares to EPM retesting.

Chapter 5: Sex Differences in Interpretive Bias and Anxiety in Adolescent Boys and Girls

1. Introduction

As reviewed in the General Introduction, interpretive bias is the perception of ambiguous information as being negative or threatening in nature. Interpretive bias is a negative cognitive bias, and has been suggested to be a vulnerability factor for anxiety disorders, as well as being important for their maintenance (Mathews & MacLeod, 2005). While interpretive bias has been found in anxious adolescents, few studies have examined sex differences in this bias during this period of life, and none have examined its potential relation to pubertal status. Examining interpretive bias and its relationship to sex during adolescence is important, as sex differences in interpretive bias may be related to the sex differences in anxiety symptomatology and prevalence of disorder. Research is also warranted on the interaction these factors may have with puberty, given that puberty is not a pathological risk factor in itself for anxiety, and interpretive bias may be a mediating factor in the puberty-anxiety relationship (Leen-Feldner et al., 2008). Having examined changes in anxiety during adolescence in rats, the aim of this chapter was to examine the presence of anxiety symptoms and interpretive bias in adolescent boys and girls, and whether these differed between the two sexes. A measure of pubertal status was also provisionally included as a way of investigating the relationship between puberty, anxiety and interpretive bias in adolescents.

In examining interpretive bias in adolescents, few studies have specifically examined potential sex differences in this bias, despite the fact that a negative cognitive style has been suggested as a factor that places adolescent girls at greater risk than same-aged boys for developing clinical anxiety or mood disorders (Hankin & Abramson, 2001; Hyde, Mezulis, & Abramson, 2008). Part of this lack of investigation may be related to the dearth of interpretive bias measures developed specifically for adolescents. Miers and colleagues (2008) recently created the Adolescents' Interpretation and Belief Questionnaire (AIBQ), which presents a set of age-appropriate written scenarios (both social and non-social) to participants and asks

whether specific positive, negative and neutral interpretations come to mind. An example of a social scenario is ‘You’ve invited a group of classmates to your birthday party but a few have not yet said if they are coming. Why haven’t they said something yet? The positive explanation was ‘They’re definitely coming; they don’t need to tell me that.’ The negative explanation was ‘They don’t want to come because they don’t like me.’ and the neutral explanation was ‘They’re definitely coming; they don’t need to tell me that.’ The scores for each category are then summed across scenarios to provide measures of interpretation bias. One of the benefits of this questionnaire measure is that it examines whether individuals interpret scenarios more negatively, or less positively, than other individuals, or both (Miers, Blöte, & Westenberg, 2011). The AIBQ also asks which of the three valenced explanations (positive, negative, neutral) the participant found to be most believable. This additional ‘belief’ question provides a measure of biased judgement, where judgement is the process by which an individual estimates the likelihood of a particular outcome (Blanchette & Richards, 2010).

Miers and colleagues’ (2008) have also been one of the few sets of researchers to examine sex differences in adolescent interpretive bias. They reported that adolescent girls (12-16 years of age, N = 33) were more negative and less positive in their interpretations of social scenarios than same-aged boys (N = 40), with no significant sex differences in these measures for non-social scenarios. Adolescent girls were also reported to have more negative beliefs in social scenarios, but not in non-social scenarios, compared to same-aged boys (Miers et al., 2008). However, given that the focus of this study was the comparison of individuals with high social anxiety scores (top 10% of the frequency distribution, N = 37) and average social anxiety scores (45-55% of the frequency distribution, N = 36), the sample did not include participants across a broader range of social anxiety scores. The study by Miers and colleagues (2008) also did not control for current depressive mood in the analyses despite its high co-morbidity with anxiety (Kessler et al., 2005; Nolen-Hoeksema & Girgus, 1994). They also did not fully investigate the effects of sex and scenario type on interpretative bias; for example, by analysing data from social and non-social situations separately, thereby preventing the researchers from asking whether

interpretation scores differed between scenario types and investigating statistically the interactions between sex and scenario type. This previous study also combined data from individuals across a relatively large age range (12-16 years).

The aim of the current study was to extend the research of Miers and colleagues (2008) by investigating sex differences in interpretation bias using the AIBQ in a non-clinical population of adolescents across a narrower age range (12-14 year old). Given the findings of Miers and colleagues (2008), it was predicted that adolescent girls would be more negative and less positive in their interpretations on the AIBQ, and more likely to believe negative interpretations, than same-aged boys, particularly for social scenarios. We also examined whether interpretation and judgement scores differed overall between social and non-social scenario types. A recent review of cognitive bias in adolescents has pointed out that relatively little is known about the content specificity of biases during this stage of life (Muris & Field, 2008). Negative interpretation bias are likely to be stronger for social compared to non-social scenarios in adolescents, as social factors, such as peer evaluation, are a major source of concern amongst this age group (Gullone & King, 1993; Westenberg, Gullone, Bokhorst, Heyne, & King, 2007). A narrow age range was selected as chronological age and the degree pubertal development are can and often are confounded (Reardon et al., 2009), meaning experiments must either statistically control for chronological age or limit the age range included. We chose 12 to 14 year old participants specifically as the rate of diagnosis of social anxiety is consistently reported to peak in early to mid-teens (Rapee & Spence, 2004). .

This study also aimed to extend the work of Miers and colleagues (2008) by adding another measure of interpretive bias, and by examining pubertal status. The additional task was adapted from one created for primary school children by In-Albon, Klein, Rinck, Becker and Schneider (2008). In their study, In-Albon and colleagues examined interpretive bias by presenting children with ambiguous social anxiety-related photos of same-aged children (potentially depicting someone popular or unpopular) and ambiguous separation anxiety-related photos (potentially depicting arrival or departure). This task was included in this study as it has the benefit of being non-verbal in

comparison to the more verbal AIBQ. In addition, it is quick to run because of it is an automated computer task, and is a child-friendly and novel measure of interpretive bias, rather than an adult measure being used on a younger sample of participants. The task also takes a different perspective from the AIBQ, with participants evaluating scenarios that other children are involved in, rather than imagining themselves in the scenarios of the AIBQ. Similarly to predictions for the AIBQ, it was hypothesised that girls may show a stronger negative bias than boys, and that social-anxiety related photos may be more negatively judged than separation-anxiety related photos in a content-specific manner. Anxiety and the rate of ambiguous photos judged negatively were also expected to correlate positively with one another.

To assess pubertal status, the Pubertal Development Scale (PDS, Peterson, Crockett, Richards & Boxer, 1988) was chosen. Although a Tanner scale completed by a trained physician is considered the 'gold standard' of assessing pubertal status (Reardon et al., 2009), this was not a viable option. The fact that the study did not take place in a clinical environment, rather it took place in a school and University, means that a physician was not easily available nor appropriate. Adolescents' self-rating themselves with the Tanner scale also presented problems; as the scale works by the participants looking at and comparing themselves to sketches of pubic hair and genital growth (as well as breast development for girls), the ethical approval and consent rates by schools, parents and even children are often lower than with questionnaire measures (e.g. Patton et al., 2004; Patton, personal communication). The PDS was therefore chosen as it benefit from being a non-graphic means of measuring the level of progression through puberty. Rather than using drawings, it presents participants with a closed set of questions on the growth spurt, pubic hair development and so on, which are answered using Likert scale responses. This test provides a total score that indicates pubertal progression known as the puberty index. As girls are known to develop sooner than boys, girls were hypothesised to have a higher pubertal index score than boys (Tanner, 1962). Correlations between the puberty index, anxiety scores and interpretive bias measures were also assessed, with positive relationships between the puberty index and these measures potentially being found for both girls and boys (Reardon et al., 2009).

To examine the relationship between anxiety and interpretive bias, the Revised Child Anxiety and Depression Scale was chosen to measure anxiety. (RCADS; Chorpita, Yim, Moffitt, Umemoto, & Francis, 2000). The RCADS is based on the separate clinical anxiety disorder sub-types and their corresponding symptoms as provided by the DSM-IV (American Psychiatric Association, 1994). Items on this scale can be divided to produce scores for depressive state and social anxiety scores, in addition to other anxiety sub-scales. We were, therefore, able to control for depression in the analyses, and, within each sex, we investigated whether individual social anxiety scores correlated with scores of negative interpretation, positive interpretation and/or judgement bias, and whether the relationships between these variables differed for social and non-social scenarios. Girls were hypothesised to have higher anxiety and depression scores than boys (following patterns of disorder at this age; Kessler et al., 2005). Higher negative interpretive bias scores on both the AIBQ and photograph task may be expected to positively correlate with anxiety scores, given that this bias is suggested to be a cognitive vulnerability factor for anxiety. The final test included in this study was the British Picture Vocabulary Scale (BPVS; Dunn, Dunn, & Whetton, 1997) The BPVS was used to assess receptive vocabulary of the participants, in order to ensure that any group differences were not the result of differences in vocabulary skills.

In summary, this study aimed to address the following research questions: a) do adolescent girls exhibit higher negative interpretation scores, lower positive interpretation scores and more negative judgement biases on the AIBQ and are girls more negative in judging ambiguous photographs than boys on the photograph task, b) are negative interpretation biases and judgement biases stronger for social compared to non-social scenarios on the AIBQ in adolescents, and is there a higher rate of negative judgements of ambiguous social compared to ambiguous separation anxiety-related photographs and c) do anxiety and interpretive bias scores from both the AIBQ and photograph task correlate with pubertal status? According to our literature searches, this is the first study to examine the potential relationship between pubertal status and interpretive bias, as well as being one of the few studies to analyse sex differences in interpretive bias.

2. Methods

2.1 Participant Information

25 boys and 22 girls aged 12-14 years old took part in the study, and were recruited in several ways. Adverts targeting the parents of 12-14 year olds were placed twice in two local papers ('The Citizen' and 'The Fife Herald'). The same adverts were also placed in the University of St Andrews Staff and Postgraduate announcements. Some participants were also recruited from years S2 and S3 of a local secondary school (Madras College), and from The Byre Theatre's youth group via information and consent letters being sent home with the children to their parents. Informed consent was gained from a parent/guardian and assent from the children. The primary language of all participants was English. Those tested out-with a school setting were paid £5 for their participation. This was not possible when children were removed from classes in school to take part, as this was deemed to be unfair to classmates not given parental consent to participate. Ethical permission was provided by the School of Psychology Ethics Committee, University of St Andrews, and Fife Education Service.

2.2 Materials and Measures

2.2.1Demographic Measures

2.2.1.1 British Picture Vocabulary Scale (B.P.V.S.; Dunn et al., 1997)

This scale was developed to assess receptive vocabulary (vocabulary for words presented orally) for children and adolescents who have grown up in an English-speaking environment. The test involves the experimenter reading out a list of words and asking the child or adolescent to choose which of four images presented for each new word depicts the meaning of that word. The words come in sets of 12, starting with the lowest set considered suitable for the age of the participant. The participant progresses through these sets of words (which increase in difficulty each time) until he/she make eight mistakes within one set or exhaust the sets of words (whichever comes first). This scale typically took 7-10 minutes to administer. The main measure provided by this scale is receptive vocabulary age in years and months (transformed into a decimal). Median reliability of the BPVS in terms of Cronbach's alpha has

been reported to be 0.93, and the test is highly valid in terms of both content and construct validity (Dunn et al., 1997).

2.2.1.2 Pubertal Development Scale (PDS; Petersen et al., 1988)

The pubertal development scale is a self-report questionnaire that assesses the presence of important morphological changes that occur as part of puberty. The questions cover changes in height (the growth spurt), body hair and skin for both sexes, as well as voice deepening and facial hair growth for boys and breast development for girls. For these morphological changes, the participants must rate whether these have not started (receiving a score of 1), barely started (score of 2), definitely started (3) or if the change described is complete (4). Girls are also asked if, and at what age or date menstruation began, and both sexes are asked about their perceived development relative to their peers. As only 4 of the 22 girls reported having not yet experienced menstruation, this information was not used in the analyses below. Further, given that the perceived timing of puberty is not of specific interest to this study and may compromise power, the responses to the question concerning development relative to peers were also not analysed.

The main measure created by this questionnaire is the puberty index – a mean of the Likert scale scores given to these morphological questions (range = 1-4). The puberty index is an average of the responses to questions regarding the growth spurt, skin changes, pubic hair changes and either voice changes for boys or breast development for girls.

Another measure created by the PDS is categorisation of respondents into five pubertal status groups using the total Likert scale scores (pre-puberty, early, mid, late and post-puberty). For boys, growth spurt, body hair, voice changes and facial hair growth responses are totalled. Boys with a score of 3 are classed as prepubertal, 4-5 with no 3-point responses ('Yes – definitely') as early-pubertal, 6-8 with no 4-point responses ('Growth completed') as mid-pubertal, 9-11 as late pubertal and a score of 12 as post-pubertal. For girls, body hair and breast development scores are totalled and menarche is considered: girls with a score of 2 with no menarche are classed as prepubertal, 3 and no menarche as early pubertal, ≥3 and no menarche as

mid-pubertal, ≤7 and menarche as late pubertal, and a total of 8 with menarche as post-pubertal.

The PDS has a median reliability of α = .77, and a median validity correlation score of .70 with interviewer's scores of adolescent's development (Peterson et al., 1988), and 0.61-0.67 correlation with a physician's Tanner scale ratings (Brookes-Gunn, Warren, Rosso & Gargiulo, 1987; in Coleman & Coleman, 2002).

2.2.2 Anxiety and Interpretive Bias Measures

2.2.2.1 Revised Child Anxiety and Depression Scale (R.C.A.D.S.; Chorpita et al., 2000)

This scale is a revision of the Spence Children's Anxiety Scale (SCAS; Spence, 1997) and was developed to assess anxiety and depression symptoms in children and adolescents according to the key criteria for being clinically diagnosed with these disorders as given by the DSM-IV. The questionnaire is comprised of five anxiety subscales (separation anxiety, generalised anxiety disorder [GAD], panic, social phobia, obsessive compulsive disorder [OCD] and a depression subscale). The questionnaire requires children to respond to 47 items, such as 'I worry when I think I have done badly at something' and 'I cannot think clearly', by circling whether they *never* (receiving a score of 0), *sometimes* (score of 1), *often* (2) or *always* (3) experience each item. Each item corresponds to one of the anxiety subscales or the depression subscale. This questionnaire typically took 5-7 minutes to complete. The measures created are total Likert scale scores for separation anxiety (range = 0-21), GAD (range = 0-18), panic (range = 0-27), social phobia (range = 0-27), OCD (range = 0-18), depression (range = 0-30), total anxiety (sum of all anxiety subscales, range = 0-111) and total anxiety and depression (range = 0-141). The total anxiety and the combined total anxiety and depression scores were not used in the analyses due to redundancy with the separately reported subscales. In terms of reliability, Cronbach's alpha scores have been reported previously as being high (e.g. Chorpita et al., 2000).

2.2.2.2 Adolescents' Interpretation and Belief Questionnaire (A.I.B.Q. – English version; Miers et al., 2008).

This questionnaire was designed by Miers and colleagues to assess interpretive bias in adolescents. The questionnaire presents a series of ten common situations for the participants to imagine themselves in, with half of these scenarios being social in context and half being non-social in context. The example scenario provided for the participants was: "A few weeks after the beginning of the new school year your teacher (mentor) asks to speak to you. Why does he or she want to speak to you?" The children are then provided with three possible responses (one positive, one negative and one neutral). For each of these, the participant must rate the likelihood of that explanation coming to mind if they were in that situation (referred to as the *mean rating of an explanation coming to mind)* and also which one of the three they found to be the most believable (referred to as the *mean belief rating*). In this example, the explanations provided were a) 'He or she wants to tell me that they are very satisfied with my work' (positive); b) 'He or she expected much better work from me and thinks that I need to work harder' (negative); c) 'He or she might want to ask me something' (neutral). This questionnaire took approximately 10 minutes for the children to complete.

Several measures were calculated from the responses to this questionnaire. For the five social scenarios, three mean Likert scale responses were created: these were the mean rating of each explanation type (positive, negative and neutral) coming to mind. These same three averages were also calculated separately across the five non-social scenarios. For the explanations chosen as the most believable for each scenario, when the positive explanation was chosen this was coded as 1, the neutral explanation as 2 and the negative explanation as 3, so that a higher score corresponded to a believing a more negative interpretation (as per Miers et al., 2008). These scores were averaged separately over the 5 social scenarios and over the 5 non-social scenarios for each participant to create two mean belief ratings.

2.2.2.3 Forced-choice Interpretive Bias Picture Task (In-Albon et al., 2008)

This task was adapted from that of In-Albon and colleagues (2008). They created a series of photograph stimuli that could be presented to test

interpretive bias in younger children as the task did not require any reading. Half of the photographs were developed to test social anxiety-specific interpretive bias, and the other half to test separation anxiety disorder-specific interpretation bias. For the social anxiety-specific photos, children are asked to judge if the child circled in the photograph is either popular or unpopular and for the separation anxiety-specific photos the children are asked whether the people in the photographs are arriving (they have just met up) or are departing (they are saying their goodbyes as they are about to leave).

A computer program was created to our specifications by technical staff in the School of Psychology in order to present In-Albon and colleagues' photographs to the participants and to record their responses. For each category of photographs, children were presented on a computer screen six unambiguous pictures (three positive, three negative) and eight ambiguous pictures, with responses to the ambiguous photographs providing the interpretive bias measures. Responses were made by pressing either the right or left mouse button, with the response words (popular/unpopular, arrive/depart) appearing and remaining on the relevant side of the screen under the photograph. The pictures were presented one at a time until a response was made or 8 seconds passed (whichever came first), and were preceded by a fixation cross presented on screen for 500 ms. The inter-trial interval was 1 second. The children were given all the instructions on screen, and told to respond as quickly and accurately as possible. Before each set of photographs there were four practice trials to familiarise the children with the stimuli and task. The order of the photograph sets and the keys used to indicate responses were counterbalanced across the participants. The order of the specific pictures within each photograph set was randomised across trials, with no more than two photographs of the same trial type being presented in a row (i.e. no more than two unambiguous positive, unambiguous negative or ambiguous photographs were shown consecutively) .This task took less than four minutes to complete.

The measures created by this questionnaire were mean reaction times to each photograph type (unambiguous positive, unambiguous negative and ambiguous, all separated by photograph set), the error rate for unambiguous

photos (in percent, referred to as the accuracy), as well as the percentage of negative responses recorded for ambiguous photographs, separated by photograph set (arrive/depart or popular/unpopular). In-Albon and colleagues (2008) found that the reliability of valence (positive, negative) for the ambiguous photos ranged from α = .79 to α =.83. In terms of validity, there were significant correlations between arrive/popular and rated pleasantness, and between higher anxiety and higher unpleasantness of departure/unpopular photographs.

2.3 Design/Procedure

The participants completed the tasks and measures in the following order: the AIBQ, the forced choice photograph task, the BPVS, the RCADS and the PDS. The interpretive bias tasks were completed before the RCADS and PDS in case filling in these personal questionnaires increased state anxiety. The participants were tested individually. Instructions for each task of the study were explained fully before commencing each one, and the participants were encouraged to ask questions if they did not understand the instructions. Once finished, each participant placed their questionnaires into an envelope marked only with their unique I.D. number and date of testing to ensure privacy and anonymity. For the photograph task, only the participant's unique I.D. and age were entered into the computer program. The experiment took up to 45 minutes in total to complete, and at the end the participants were fully debriefed.

2.4 Statistical Analyses

To check that the paid and unpaid participants could be treated as one whole group, a multivariate analysis of variance (MANOVA) was performed. Participant group (paid or unpaid) and sex were the between-subjects factors, and the within-subjects variables were chronological age, receptive vocabulary age and the puberty index score from the P.D.S. A second MANOVA was run to compare the chronological age, receptive vocabulary age and puberty index scores between boys and girls (the between-subjects variable), collapsed across the paid and unpaid participant groups.

For the R.C.A.D.S. questionnaire, a MANOVA was used to examine any possible sex differences in the anxiety subscale and depression scores. Sex was the between-subjects factor, and the within-subjects variables were social phobia, total anxiety and the depression subscale scores. To test the hypothesis that anxiety and pubertal status are positively related, the relationship between the puberty index and both total anxiety and social phobia scores were analysed using one-tailed Pearson's r tests of correlation. These were performed separately for boys and girls, given that, on average, girls tended to have progressed further through puberty than boys as detailed in the results below.

For the A.I.B.Q., a repeated measures ANOVA was performed on the mean explanation coming to mind ratings, with sex as a between-subjects variable, and scenario type (social, non-social) and explanation valence (positive, negative) as the within-subjects variables. Neutral explanations were not included in this ANOVA to allow for direct comparison with Miers et al.'s study (2008), as neutral responses were removed from their analyses. Any sex differences in positive and negative biases were therefore examined in this test. Significant interactions were further examined using simple effects tests. Sex differences in the mean belief ratings were analysed in a separate repeated measures ANOVA, with sex as a between-subjects factor and scenario type as the within-subjects factor.

To examine whether there was a positive relationship between interpretive bias and anxiety, one-tailed Pearson's r tests for correlation were run as appropriate between total anxiety, social phobia and the mean ratings of positive and negative explanations coming to mind, as well as the mean belief ratings. These measures from the A.I.B.Q. were also entered into Pearson's r tests of correlation with puberty index scores. All of these correlations were separately calculated for scenario type, and the puberty index calculations were again carried out separately for boys and girls.

For the photograph task, a repeated measures ANOVA was used to examined reaction times between the sexes, trial types (ambiguous, positive/negative unambiguous) and photograph sets (arrive/depart and popular/unpopular). A second repeated measures ANOVA was run where reaction times were replaced by accuracy (the percentage of unambiguous

photographs characterised correctly). Ambiguous trials were removed from the factor trial type in this test.

Sex differences in interpretive bias were investigated by examining the percentage of negative responses to ambiguous photographs in a repeated measures ANOVA. Sex was therefore a between-subjects factor and photograph set a within-subjects factor.

Data reported in brackets are means ± SEMs. SPSS version 17 was used to analyse the data, with an alpha level of $\alpha < .05$ was used throughout, except where multiple correlations were run when the alpha level was adjusted by dividing $\alpha < .05$ by the number of correlations calculated. Effect sizes are reported as η_p^2. All 47 participants were included in the analysis with the exception of the photograph task, where one female participant failed to press any response keys when presented with the photographs and was therefore omitted.

3. Results

3.1 Cohort Comparisons

Both sets of participants were compared (paid versus unpaid) using a MANOVA, and there were no significant differences between these two groups in terms of chronological age ($F_{1, 45} = 0.41$, $p = .840$, $\eta_p^2 = .001$), receptive vocabulary age ($F_{1, 45} = 1.09$, $p = .303$, $\eta_p^2 = .025$) or puberty index ($F_{1, 45} = 1.89$, $p = .177$, $\eta_p^2 = .042$). There was also no interaction between participant group and sex for either chronological age ($F_{1, 45} = 0.85$, $p = .362$, $\eta_p^2 = .019$), receptive vocabulary age ($F_{1, 45} < .001$, $p = .984$, $\eta_p^2 < .001$), or puberty index scores ($F_{1, 45} = 0.77$, $p = .386$, $\eta_p^2 = .017$). Therefore, in all the analyses detailed below the participants were considered as one whole group.

3.2 BPVS and RCADS scores

Receptive vocabulary age did not differ between the sexes ($F_{1, 45} = 1.37$, $p > 0.05$, $\eta_p^2 = .029$; **Table 5.1**). In addition, none of the participants had a receptive vocabulary age lower than expected for his/her chronological age. Girls had higher total anxiety scores than boys ($F_{1, 45} = 4.68$, $p = .036$, $\eta_p^2 = .094$) and higher social phobia scores than boys ($F_{1, 45} = 4.46$, $p = .040$, $\eta_p^2 =$

.090; **Table 5.1**). The main effect of sex on depressive subscale was not significant ($F_{1, 45} = 2.36$, $p = .131$, $\eta_p^2 = .050$).

Table 5.1 BPVS and RCADS scores for girls and boys (means ± SEMs). * = $p < .05$ indicating significant main effect of sex.

		Mean ± SEM
Receptive vocabulary age (years)	Girls	13.6 ± 0.2
	Boys	13.4 ± 0.2
RCADS: total anxiety score	Girls	35.9 ± 3.6
	Boys	24.8 ± 3.2*
RCADS: social phobia score	Girls	14.0 ± 1.2
	Boys	10.3 ± 1.1*
RCADS: depression score	Girls	9.2 ± 1.0
	Boys	7.0 ± 0.9

3.3 PDS Scores and Correlations to RCADS

As expected, girls tended to be more developed than boys as measured by the puberty index ($F_{1, 45} = 3.60$, $p = .064$, $\eta_p^2 = .074$; boys: 2.4 ± 0.1; girls: 2.7 ± 0.1). The responses to the PDS were also used to categorise the participants into the five puberty groups. No participants matched the criteria for being pre-pubertal. In the early puberty group, there were 7 boys and no girls. In the mid puberty group, there was 13 boys and 3 girls. In the late puberty group, there were 5 boys and 2 girls, and in the post-puberty group there were no boys and 17 girls. Given that girls are known to progress through puberty earlier than boys, these results suggest the questionnaire successfully located morphological differences in the pubertal development of boys and girls of the same age.

The hypothesis that anxiety might be positively correlated with the puberty index was assessed using Pearson's r tests of correlation. However, there were no significant correlations between the puberty index and total anxiety for either boys ($r = -.142$, $p = .249$) or girls ($r = .162$, $p = .235$). The same was true when social phobia was entered into the correlation in place of

total anxiety for either boys or girls (boys: r = -.016, p = .470; girls: r = -.222, p = .161).

3.4 AIBQ scores

3.4.1 Negative and positive interpretation scores

For the mean ratings of positive and negative explanations coming to mind, the three-way interaction between sex, valence (positive, negative) and scenario type (social, non-social) was not significant ($F_{1,45} = 0.01$, $p = .909$, $\eta_p^2 < .001$), nor was the interaction between scenario type and sex ($F_{1, 45} < .001$, $p = .993$, $\eta_p^2 = .001$). There was a significant interaction between sex and valence ($F_{1, 45} = 4.08$, $p = .049$, $\eta_p^2 = .083$; **Table 5.2**), due to girls being more likely than boys to bring negative interpretation to mind ($p < .01$). Positive interpretations were not significantly different between girls and boys ($p > .01$), and no other pair-wise comparisons were significant. When depressive score was included as a co-variate, the interaction between sex and valence was reduced to a trend ($F_{1, 44} = 3.38$, $p = .073$, $\eta_p^2 = .071$).

There was a significant interaction between scenario type and valence ($F_{1, 45} = 35.90$, $p < .001$, $\eta_p^2 = .444$; **Figure 5.1**). Positive interpretations were significantly more likely to come to mind in non-social than in social scenarios ($p < .001$), and positive interpretations were significantly more likely to come to mind than negative interpretations in non-social scenarios ($p < .01$). No other pair-wise comparisons were significant. The interaction between scenario type and valence remained significant when depressive score was included as a co-variate ($F_{1, 44} = 6.28$, $p = .016$, $\eta_p^2 = .125$).

Table 5.2 Mean rating of positive and negative explanations coming to mind, collapsed across scenario type (means ± SEMs). * = p < .05 indicating a significant main effect of sex.

		Mean ± SEM
Negative interpretation score	Girls	2.9 ± 0.6
	Boys	2.6 ± 0.7*
Positive interpretation score	Girls	3.2 ± 0.5
	Boys	3.2 ± 0.6

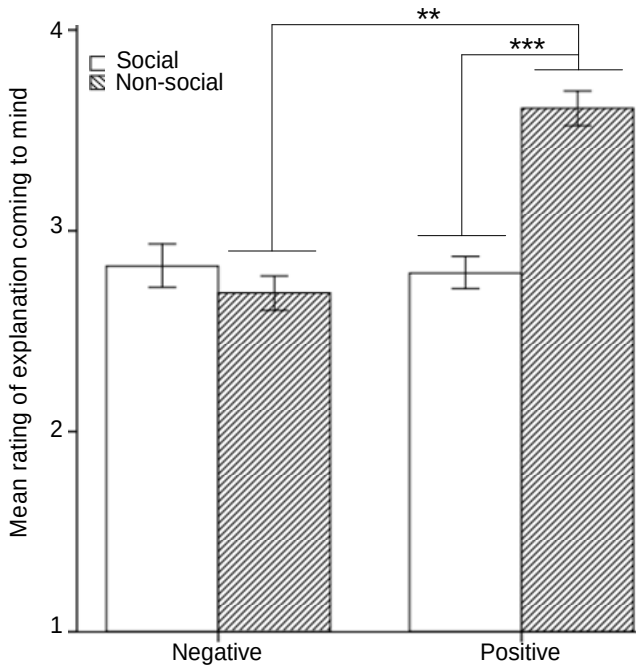

Figure 5.1 Mean rating of negative and positive explanations coming to mind in the social and non-social scenarios of the AIBQ. (means ± SEMs). ** indicates p≤ .01, *** indicates p < .001 in post-hoc comparisons.

3.4.2 Mean belief rating

For mean belief rating, the main effect of scenario was significant ($F_{1, 45}$ = 104.91, p < .001, η_p^2 = .700), with mean belief ratings for social scenarios being significantly higher, and therefore more negative, than the mean belief ratings for non-social scenarios (social scenarios: 2.3 ± 0.1, non-social scenarios: 1.6 ± 0.1). The main effect of scenario type remained significant when depressive score was included as a co-variate ($F_{1, 44}$ = 28.55, p < .001,

η_p^2 = .393). There was no main effect of sex on the belief rating ($F_{1, 45}$ = 2.07, p = .158, η_p^2 = .044; boys: 1.7 ± 0.3; girls: 1.8 ± 0.3), and the interaction between scenario type and sex was not significant ($F_{1, 45}$ = 0.02, p = .883, η_p^2 < .001).

3.4.3 Correlations between RCADS social phobia scores, the Puberty Index and AIBQ scores

For boys, the RCADS social phobia score positively correlated with the mean score for negative interpretations coming to mind in both social scenarios (r = .688, p <.001) and in non-social scenarios (r = .578, p = .002). In comparison, this relationship was not significant for girls in either social (r = .301, p = .174) or non-social scenarios (r = .295, p = .183). There were no significant correlations between social phobia scores and the mean rating of positive interpretations for boys in either social (r = .150, p = .473) or non-social scenarios (r = .323, p = .115) or for girls in either social (r = .078, p = .729) or non-social scenarios (r = .253, p = .255). For both scenario types, social phobia scores did not correlate with the mean belief rating for either boys (social: r = -.098, p = .641; non-social: r = .212, p = .310) or girls (social: r = -.056, p = .806; non-social: r = .033, p = .884).

To examine the hypothesis that pubertal status and interpretive bias may also be related, correlations were used to examine the relationship between the puberty index and the AIBQ results. For both boys and girls, there were no significant correlations between the puberty index and either the mean rating of positive or negative explanations for both social and non-social scenarios, and no correlation between puberty index and the mean belief rating for social and non-social scenarios (**Table 5.3**).

Table 5.3 Summary of correlations between the puberty index score and the AIBQ measures interpretive bias measures for boys and girls (mean ± SEMs).

		Mean correlation with puberty index
Social: positive explanation rating	Girls	r = .107, p = .317
	Boys	r = .282, p = .086
Social: negative explanation rating	Girls	r = .-.046, p = .419
	Boys	r = -.207, p = .161
Social: mean belief rating	Girls	r = .221, p = .162
	Boys	r = .355, p = .041‡
Non-social: positive explanation rating	Girls	r = -.101, p =. 327
	Boys	r = .007, p = .488
Non-social: negative explanation rating	Girls	r = -.205, p = .180
	Boys	r = -.144, p = .287
Non-social: mean belief rating	Girls	r = .080, p = .361
	Boys	r = .096, p = .234

‡ This is not a significant correlation as $\alpha \leq .008$

3.5 Forced Choice Photograph Judgement Task

3.5.1 Interpretive Bias

To examine the hypothesis that there may be a sex difference in interpretive bias, a repeated measures ANOVA was employed to examine sex differences in the categorisation of ambiguous photographs, with sex as the between-subjects factor and photograph set as the within-subjects factor. Collapsing across photograph set, there was no overall sex difference in the percentage of ambiguous photographs that were negatively characterised ($F_{1, 44} = 0.06$, $p = .804$, $\eta_p^2 = .001$). There was a main effect of photograph set however ($F_{1, 44} = 17.30$, $p < .001$, $\eta_p^2 = .282$), with more of the ambiguous arrive/depart photographs being characterised as depicting departure compared to ambiguous popular/unpopular photographs depicting someone unpopular (ambiguous photos as departure: 68.4 ± 2.9%, ambiguous photos as

unpopular: 50.7 ± 3.3%). There was no interaction between sex and photograph set however ($F_{1, 44}$ = 0.28, p = .599, η_p^2 = .006).

3.5.2 Reaction Times

A repeated measures ANOVA was used to examine any sex differences in reaction times. The within-subjects factors were the photograph set (arrive/depart, popular/unpopular) and the trial type (unambiguous positive, unambiguous negative and ambiguous), and the between-subjects factor was sex. There were significant main effects of both photograph set ($F_{1, 45}$ = 49.60, p < .001, η_p^2 = .524) and trial type ($F_{2, 90}$ = 24.39, p < .001, η_p^2 = .351) on reaction times. Arrive-depart photographs were responded to significantly faster than popular-unpopular photographs (arrive/depart photographs: 1906.8 ± 116.1 milliseconds, popular/unpopular photographs: 2471.9 ± 144.6 msecs). Post-hoc comparisons revealed that ambiguous photographs were responded to more slowly than both positive (p < .001) and negative (p < .001) unambiguous photographs (unambiguous positive photos: 1844.3 ± 111.9 msecs, unambiguous negative photos: 2071.7 ± 136.6 msecs, ambiguous photos: 2652.0 ± 172.6). There was no interaction between trial type and photograph set ($F_{2, 90}$ = 0.90, p = .409, η_p^2 = .020) and no three-way interaction between sex, photograph set and the trial type in terms of reaction times ($F_{2,90}$ = 1.04, p = .357, η_p^2 = .023). There was also no main effect of sex ($F_{1, 45}$ = 0.18, p = .674, η_p^2 =.004) and no interactions between sex and either photograph set ($F_{1, 45}$ = 2.06, p = .158, η_p^2 = .044) or trial type ($F_{2, 90}$ = 0.44, p = .646, η_p^2 = .010).

3.5.3 Accuracy

Accuracy (the percentage of unambiguous photographs responded to correctly) was investigated using a separate repeated measures ANOVA. Sex was a between-subjects factor, with trial type) and photograph set as within-subjects factors. There were no significant differences between boys and girls regardless of photograph set and trial type ($F_{1, 44}$ = 1.39, p = .245, η_p^2 = .031). There was a main effect of trial type however ($F_{1, 44}$ = 29.49, p < .001, η_p^2 = .401), with positive photographs (unambiguous popular and arrival) producing a significantly higher accuracy rate than negative (unambiguous unpopular

and departure) photographs (unambiguous positive photos: 83.9 ± 1.5 % correct, unambiguous negative photos: 95.1 ± 1.4 % correct). There was also a significant main effect of photograph set ($F_{1, 44}$ = 39.19, p < .001, η_p^2 = .471), with arrive/depart photographs producing higher accuracy rates than the popular/unpopular photographs (arrive/depart photos: 96.5 ± 1.4 % correct, popular/unpopular photos: 82.5 ± 1.7 % correct). Following from this, there was also an interaction between trial type and photograph set for accuracy ($F_{1, 44}$ = 82.39, p < .001, η_p^2 = .652), which appears to be the results of positive pictures for both photographs sets were rated more accurately, with negative photographs in both sets being rated less accurately. There were no interactions between sex and trial type ($F_{1, 44}$ = 1.38, p = .246, η_p^2 = .030), sex and photograph set ($F_{1, 44}$ = 0.31, p = .582, η_p^2 = .007) and no three-way interaction between sex, trial type and photograph set ($F_{1, 44}$ ¸.001, p = .997, η_p^2 < .001).

4. Discussion

The aim of this study was to assess interpretive bias in young adolescent boys and girls, examining sex differences in interpretive bias, and the potential relationships between interpretive bias, anxiety and pubertal status. The results showed that the mean negative interpretation score was higher (i.e. negative interpretations were rated as more likely to come to mind) for girls than boys, regardless of scenario type, while the sexes did not differ in positive interpretation scores or in which scenarios they most believed. For both sexes combined, mean positive interpretation scores were lower (i.e. positive interpretations were less likely to come to mind) for social scenarios, and adolescents were more likely to believe negative interpretations of social scenarios, compared to non-social scenarios. In boys, individual scores of social phobia positively correlated with the mean score for negative interpretations coming to mind in both types of scenario, indicating that anxious males scored high on negative interpretations regardless of scenario type. Potential reasons for the lack of similar correlations in girls are discussed below. The forced choice photograph task did not reveal any sex differences in interpretive bias, although reaction time and accuracy findings confirmed that the task was understood and carried out correctly. Pubertal

status, as measured by the puberty index, did not correlate with anxiety scores or with interpretive bias measures for either boys or girls. Potential reasons for these findings are discussed below. In summary, these results show that adolescent girls were, on average, more likely than same-aged boys to bring negative interpretations to mind, and both male and female adolescents exhibited content-specific negative interpretation and judgement biases when confronted with ambiguous social scenarios.

4.1 Sex Differences in Interpretive Bias

The finding that girls exhibited a stronger negative interpretation bias than boys (as measured on the AIBQ) is in line with a small number of studies that have examined sex differences in cognitive biases in adolescents (e.g. Bell-Dolan, 1995; Hankin & Abramson, 2002; Miers et al., 2008; van Beek & Dubas, 2008; Vasey, El-Hag & Daleiden, 1996). These studies have found similar sex differences using a range of tests, including the processing of threatening and neutral words (Vasey et al., 1996), the interpretation of videos of peer-interactions (Bell-Dolan, 1995), the assessment of ambiguous facial expressions (van Beek & Dubas, 2008) and inferring casual explanations from negative events (Hankin & Abramson, 2002). As cognitive biases have been suggested to enhance the susceptibility to anxiety disorders and aid their maintenance (Mathews & McLeod, 2005), sex differences in biases such as interpretive bias could be instrumental in the increased susceptibility of adolescent girls to anxiety disorders relative to boys of the same age. A number of factors may potentially underlie the emergence of sex differences in cognitive biases during adolescence, including the pressure of gender norms, changes in social interactions, and physical changes in anatomy and hormone levels (Hyde, et al., 2008; McLean & Anderson, 2009; Nolen-Hoeksema & Girgus, 1994; Reardon et al., 2009; Rutter, Caspi, & Moffitt, 2003). Given that the sex difference in negative interpretation on the AIBQ was reduced to a trend when depressive score was included as a co-variate in the analysis, interpretation bias does appear to be partially moderated by current depressive mood, with depression being another common disorder in adolescence, especially in girls (Kessler et al., 2005; Nolen-Hoeksema & Girgus, 1994).

Comparing our AIBQ findings to those of Miers and colleagues (2008), Miers' paper reported that females were less positive, and had more negative beliefs, than boys when responding to social scenarios, although not when presented with non-social scenarios. In comparison, the current study did not show that girls have a higher mean score for positive interpretations (i.e. girls were not less positive than boys), nor that adolescent girls were more likely than boys to believe negative explanations, for either social or non-social scenarios. In addition, Miers and colleagues reported that girls were more likely than boys to bring negative interpretations to mind for only social scenarios, while the interaction between sex and scenario type was not significant for negative interpretation scores in the current study. The differing results between the two studies are not obviously due to differences in sample size or the age of the participants involved. The study by Miers and colleagues (2008) had a smaller sample of control subjects (16 girls, 20 boys) than the current study (22 girls, 25 boys), but did have a larger total sample size due to the inclusion of a set of participants that scored very high on social anxiety (17 girls, 20 boys). The mean age of subjects across the two studies was very similar (this study: mean = 13.4 years; Miers et al., 2008: mean = 13.6 years), although the study by Miers and colleagues (2008) included a broader age range of participants (11-16 years old). Other methodological differences that could have influenced the results include the exclusion of participants with low social anxiety scores in the study by Miers and colleagues (2008) and the nationality of the participants (this study: British participants; Miers et al., 2008: Dutch participants). However, regardless of the reasons for the different findings, our data highlight the fact that the likelihood of negative interpretations coming to mind can vary *independently* from the propensity to favour positive interpretations and from the likelihood of believing in negative outcomes.

In comparison to the AIBQ findings, interpretive bias was not found to be significantly different between the sexes on the forced choice photograph task, despite reaction time and accuracy results confirming that ambiguous photographs took the longest response time to respond to, and unambiguous photographs were accurately categorised. In fact, the percentage of ambiguous photographs characterised negatively across the whole

experiment was 59.5%, with 50% indicating a random response. This suggests that the task was not successful in locating an interpretive bias, especially as interpretive biases were detected using the AIBQ. This inability to detect interpretive bias may have been due to a lack of engagement of the participants with the task. The photographs were of much younger children (primary school age) rather than of fellow adolescents, so the participants would have been less likely to identify with the children photographed. In addition, separation anxiety is a disorder of early childhood (Kessler et al., 2005), so a content-specific bias might not be expected in participants aged 12-14years old for the arrival-departure photographs.

Another contributing factor to not finding sex differences in interpretive bias with the photograph task may have been the perspective taken by the participants: the AIBQ required the participants to imagine themselves in each scenario, while this task involved responding to the situation of another child (i.e. from a third-person perspective). Another study has found girls to be more biased than boys when using a third-person perspective: Bell-Dolan (1995) found that girls judged more videos of ambiguous interactions between peers to be hostile in comparison to boys. However, not only were the children in the video similarly aged to the participants unlike the photographs in our study, the videos may be more engaging than the purely visual photograph task. While the photographs have been shown to be reliable, valid and useful in younger children (In-Albon et al., 2008), adolescent-specific material may be required for this task to be similarly sensitive to interpretive biases in older children. The results of this task were therefore not further analysed in terms of content-specificity and the relationship with anxiety and the puberty index.

4.2 Content-specificity of Interpretive Biases

In our study, the likelihood of positive explanations coming to mind was significantly higher for non-social scenarios than social scenarios regardless of the sex of the participant, with no difference in the likelihood of negative explanations coming to mind across the two scenarios. This result provides further evidence for the fact that patterns of positive and negative interpretations can vary independently from one another. While this seeming

independence of positive and negatives biases requires replication using a range a measures, the finding that interpretation and judgement biases exhibit content specificity in a non-clinical sample of early to mid-adolescents is novel and is also consistent with evidence that social issues, such as peer evaluation, are a key source of concern in this age group (Westenberg et al., 2007). Miers and colleagues (2008) analysed their data from social and non-situations separately and were, therefore, not able to ask whether interpretation scores differed between scenario types. Although relatively little is known about the content specificity of cognitive biases during adolescence (Muris & Field, 2008), a small number of previous studies have suggested that socially anxious children and adolescents exhibit a stronger negative judgement bias when presented with ambiguous social scenarios compared to non-social scenarios (e.g. Rheingold et al., 2003; Spence, Donovan, & Brechman-Toussaint, 1999), but such findings have been inconsistent (e.g. Muris et al., 2000; reviewed by Alfano, Beidel, & Turner, 2002).

4.3 Correlations between Anxiety and Interpretive Bias

With regards to sex differences in interpretive bias and anxiety, studies of non-clinical populations of adolescents have a) reported that interpretation bias is significantly related to anxiety disorder status for girls but not for boys (Cannon & Weems, 2010), b) failed to find a moderating effect of sex on the relationship between cognitive bias and self-reported anxiety (e.g. Weems, Costa, Watts, Taylor, & Cannon, 2007), or c) failed to reported the effects when sex is included in such analyses (e.g. Vassilopoulos & Banerjee, 2008). In our study, social phobia scores correlated positively with the number of negative explanations coming to mind in both social and non-social scenarios in boys, but not in girls, although we remain cautious about interpreting these analyses due to the limitations of sample size. The lack of significant correlations in girls was not due to a lack of variance in social anxiety scores or negative interpretation scores in girls, as the variance in each of these measures was similar for the two sexes, and we have no evidence for poorer reporting accuracy on the anxiety scale in girls than boys. The potential moderating effect of sex on the relationship between interpretation bias and anxiety symptoms therefore requires further investigation.

4.4 Puberty between Anxiety and Interpretive Bias

The puberty index did not correlate with total anxiety, society phobia or any of the interpretive bias measures of the AIBQ (mean scores for positive and negative rating coming to mind, and mean belief scores). While we can find no research examining the potential relationship between pubertal status and interpretive bias, anxiety symptomatology and pubertal status have been found to positively correlate in girls, and a similar trend has been recorded in boys, who are less often included in such experiments (see Reardon et al., 2009 for a review).

The fact that correlations between puberty index and either anxiety or interpretive bias were not found does not preclude the fact that they do exist. The lack of a relationship between the puberty index and the other variables may be due to a relatively low sample size, especially given that relationship between anxiety and interpretive bias are often found only in larger scale studies. For example, Ge and colleagues (2006) found a relationship between anxiety and pubertal status, but with a total 400 male and 467 female participants. Unfortunately effect sizes have gone unreported in such studies preventing the assessment of power, although a large sample size would be required should the effect size of pubertal status on anxiety or interpretive bias be found to be small. We therefore cannot make any conclusions on such relationships due to our presumed lack of power, although further investigation as to the possibility of interpretive bias mediating the link between puberty and anxiety is warranted.

4.5 Conclusion

In summary, early to mid-adolescent girls exhibited a stronger negative interpretation bias than same-aged boys, which could play a role in increasing the vulnerability of adolescent girls to anxiety disorders relative to boys. Early to mid- adolescents of both sexes also responded less positively to social scenarios, and were more likely to believe negative interpretations of social scenarios, compared to non-social scenarios. Gaining an understanding of the aetiology of anxiety disorders has enormous potential for improving the development of sex- and age-specific interventions and treatments (Rapee,

Schniering, & Hudson, 2009; Sauter, Heyne, & Westenberg, 2009). For instance, training individuals to engage in positive interpretation bias might be most productively targeted towards social scenarios. Such training has recently been shown to be successful in decreasing negative affect in adolescents (e.g. Lothmann, Holmes, Chan, & Lau, 2011; Salemink, & Wiers, 2011; Vassilopoulos, Banerjee, & Prantzalou, 2009). Larger scale studies are required in future to characterise the relationship between anxiety and pubertal status more fully, and to examine whether interpretive bias has any involvement in mediating this relationship. This work could also be further aided by the creation of more adolescent-specific interpretive bias assessments and materials.

Chapter 6: General Discussion

1. Summary

The aim of this thesis was to assess sex differences and changes in anxiety-related behaviour across adolescent male and female rats, and to assess symptoms of anxiety and their relation to interpretive bias in adolescent boys and girls. Both species were examined in the hope of moving towards better reconciliation of both fields of research, and improved translational validity across anxiety-related measures in two fields of research. Chapter 2 began by assessing the effects of the rat's ovarian cycle on elevated plus-maze behaviour, as any significant effects on the behaviour of adult female rats would have to be considered in the design of later studies. This would have included lavaging and assessing the cycle phase of adult females, lavaging (but not assessing) the irregular or not-yet-started cycles of adolescent females, and giving all male subjects lavage-like handling. While the gonadal hormones of the female's oestrous cycle are undoubtedly related to performance on common tests of anxiety-like behaviour, cycle effects were not detected in our Lister-hooded rats. This issue was therefore not further addressed, although should cycle differences be found using a standard protocol, then care should be taken to examine whether adolescent rats are affected differently by lavage-like handling compared to adult rats.

Chapter 3 examined the patterns of anxiety-like behaviour across adolescence and adulthood, with adolescents generally found to have lower levels of exploration than adult rats. Across the age groups, exploration on the emergence test, open field and elevated plus-maze generally increased. While the results of each behavioural test correlated with one another, indicating that they measure a similar type of behaviour or experience, the problem of how to use several tests without the test order or testing schedule affecting the behaviour of the rats remains.

The issue of testing order and schedule was addressed in Chapter 4, by examining how behaviour on the EPM changes when preceded with another behavioural test – this time the locomotor box. Exposure to this novel test generally increased open arm exploration on the EPM. Although sex

differences were found on the EPM (in contrast to chapter 3), no differences in adult and adolescent behaviour on the two tests were found, and age did not interact with order. In other words, the order of testing did not appear to affect adult and adolescent rats differently. This is in contrast to other work that has reported adolescents to be more sensitive to pre-test experiences than adult rats (Doremus-Fitzwater et al, 2009). As discussed earlier, these inconsistencies may be a product of methodological differences, and more work is required to assess which behavioural tests and what type of testing schedules may differently affect adolescent and adult, or male and female, behaviour.

Chapter 5 set out to examine sex differences in interpretive bias in adolescent boys and girls. Girls were found to be more negative but not less positive than boys when presented with the ambiguous scenarios of the AIBQ. Social scenarios were also interpreted more negatively in general than non-social scenarios, which fits with the salience of the social domain during this period of life. While pubertal status was not found to be related to anxiety or to interpretive bias in this study, this remains an important line of research given that interpretive bias could potentially mediate the relationship between puberty and anxiety.

In summary, adolescence remains an important time in the emergence of anxiety disorders in humans, when changes in performance on anxiety-related tasks and in symptoms are also found in rats and in humans respectively. Many gaps in our knowledge still exist for both species; detailed below are some important options for further research.

2. Future Directions

2.1 Rodent Research

More research is warranted on how exploratory behaviour changes in purported tests of anxiety-related behaviour across adolescence and into adulthood. While a within-subjects design is ideal for tracking changes in the behaviour of an individual rat across this period of development, repeat exposure to tests such as the EPM produce a different profile of behaviour, making it a less suitable approach for such studies (e.g. Dawson et al., 1994; Espejo, 1997; File et al., 1993; Griebel et al., 1993; Rodgers et al., 1992).

Using a between-subjects design, care must be taken when deciding the number of tests, their order and the schedule of testing, balanced against the width of age ranges included in each age group. The findings we have reported require further replication, including replication in other laboratories, to support their reliability. Such research could be further added to by using a single test such as the EPM, or multiple tests could again be used, with sensitivity given to the effects testing order may have, and how testing schedules are balanced against the age ranges used for each sub-group of rat.

One problem remains with unconditioned tests of anxiety-like behaviour though, and that is their interpretation. The interpretation that females who enter and remain on the open arms of the EPM for example, requires various assumptions: that both sexes are equally motivated to explore, that the animals seek to explore the open arms as they are curious or neophilic, and that anxiety is preventing males from exploring them. An animal may enter an open arm of the elevated plus-maze or the centre of the open field because it is actively seeking a way of escape or because it is experiencing something akin to low-anxiety. Many motivations of the rat could also be having a role to play in an animal's performance on this test. For example, hunger, thirst and sexual receptivity may be motivations for an animal to explore new areas and be less averse to risk. While Chapter 2 of this thesis did not reveal significant effects of the oestrous cycle on EPM behaviour, this cannot be fully discounted as a potential explanation for the sex differences commonly reported on this test. Males and females may be differently motivated: while an adult male is always fertile and capable of reproducing, females are only fertile for short periods of their cycle, and this may play a role in their differing behaviour. While water and food were provided ad libitum to our rats so preventing any significant hunger and thirst, these factors may play more of a role in studies where animals are out of the housing room for longer periods of time to complete longer batteries of tests, or for animals that must be at least partly deprived of food or water for the purposes of administering drugs. Separating these potential explanations and motivations is not possible by behavioural means alone, but is required if the

behaviours reported using the EPM are to be more confidently interpreted as ‘anxiety-like’.

At first hand, the fact that males appear to exhibit less anxiety-like behaviour than females in the EPM is at odds with the hypothesis that as sole care-givers to offspring, female rats (like other mammals) may be generally more cautious and aversive to risk then their male conspecifics. This again raises the question of whether the EPM is measuring anxiety, and the behaviour of rats of the opposite sex or of different ages also remains difficult to interpret. Johnson and File (1991) themselves cautioned that conclusions about sex differences in anxiety cannot easily be drawn from sex differences in open arm activity, as the EPM might not measure the same variables in male and female rats. In the wild, adult female rats typically remain in or near to their home burrows while adult male rat disperse (Calhoun, 1963), and as sole care-givers to their young may, for instance, use a different spatial strategy to explore novel environments than males. Female rats might be predicted to exhibit a higher motivation to learn about the immediate environment than males, if females are more likely than males to remain in the local area surrounding their natal burrow system. Learning features of the local environment might not benefit young males that will disperse to new territories, while females must know their locale well to protect and feed their young. The fact that males peeked onto the open arms more than females in Chapters 3 and 4, but females explored the open arms more than males may be a difference of exploration strategy, rather than one of anxiety. Male and female behaviour on the EPM has also been found to load differently when assessed using factor analysis (e.g. Fernandes et al., 1999; File, 2001), with the primary factor for males containing measures considered by these researchers to be anxiety-like, while for females the primary factor is one of locomotion. The behaviour of adult and adolescent rats has however been found to load similarly (Doremus, Varlinskaya & Spear, 2006). Jones, Braithwaite and Healy (2003) do caution that as a one off event, dispersal may not be a strong selective force on spatial ability however.

One strength and weakness of the EPM is that it is a one-trial, unconditioned test; while it does not require training, it can only be used once as it relies on the novelty of the apparatus. When used in repeat testing, not

only do rodents spend less time on the open arms on their second exposure to the EPM (e.g. Dawson, Crawford, Stanhope, Iversen & Tricklebank, 1994; Espejo, 1997; File, Zangrossi, Viana & Graeff, 1993; Griebel, Belzung, Misslin & Vogel, 1993; Rodgers et al., 1992), but reasons for such changes in behaviour (such as habituation of learning that there is no escape) are difficult to parse.

Healy, Braham and Braithwaite (1999) suggest that the traditional spatial task, the Morris Water Maze (a conditioned test where an animal learns the position of a submerged platform to exit the water over a series of trials) may measure something more akin to stress or anxiety than to spatial learning, given that females continue to swim the walled perimeter of the apparatus while males quickly swim to the platform, despite both sexes having successfully learnt the location of this platform (e.g. Beiko, Lander, Hampson, Boon, & Cain, 2004; Harris, D'Eath & Healy, 2008). Females would thus be considered to be showing more anxiety-like behaviour than male rats, rather than less. Anxiolytic drugs can eliminate sex differences in performance on this task by reducing thigmotaxis in female rodents (e.g. Galea Saksida, Kavaliers & Ossenkopp, 1994). However, behaviours on this task also remains subject to issues of interpretation, and in terms of pharmacological validation it is not clear whether such anxiolytics are affecting only anxiety-like behaviour and not spatial cognition itself in female rats, an effect that could be obscured by a floor effect in males who may already have optimally low latencies in reaching the platform. This test may also not necessarily have greater utility than the EPM for developing anxiolytic compounds in preclinical research, especially as only the performance of females is affected by anxiolytic administration.

Despite these issues, the behaviour of rats on the EPM and differences between the sexes and the ages remain of interest regardless of their interpretation. In terms of pharmacological research, the behaviours measured on these tests remain predictive of the effects of most anxiolytic and anxiogenic drugs. However, it is not clear whether such behaviours index 'anxiety', or if 'anxiety' is just one component of what is being measured. One approach to aid interpretation of behaviour is to examine stress hormone profiles of rats before, during and after exposure to such tests. However,

physiological measures can suffer from similar problems of interpretation in that high corticosterone could index high anxiety, high excitement or a mixture of both. A new type of behavioural test has been developed that has addressed some of these problems however – a tests of interpretive bias in rats.

In 2004 Harding, Paul and Mendl reported an experiment where the affective response of rats to changes in housing conditions was assessed using a conditioned task that required the judgement of ambiguous sounds. The rats were trained that one tone represented the presentation of a food pellet, while another tone represented an aversive event (white noise). Rats were either in consistent or unpredictable housing conditions (including tilting the cage, a non-familiar intruder in their cage, wet bedding and so on). The latter conditions are commonly reported to produce a depressed-like state in rodents. Subsequently when presented with ambiguous tones in the middle frequencies between the positive and negative tones, unpredictably housed rats were slower to respond in general and made fewer lever presses for ambiguous tones that were close to the positive training tone in comparison with predictably housed animals. The unpredictably-housed rats therefore showed a negative bias in interpreting the ambiguous noise stimuli. Similar results have been reported when training rats and starlings to respond to colours instead of sounds, where white and black represent positive/negative, outcomes with varying greyscale probes providing ambiguous probe trials (Bateson & Matheson, 2007; Burman, Parker, Paul & Mendl, 2009).

Rats have been shown to be more negatively biased when exposed to conditions already known to decrease exploration on common tests of anxiety-related behaviour. Burman and colleagues (2009) trained rats to discriminate between black and white-lidded food pots in equidistant probe locations in either low light or bright light conditions, the latter being known to decrease open arm exploration in the EPM (e.g. Hogg, 1996; Mora et al., 1996). Half the rats then ran the ambiguous test trials in the opposite light conditions under which they were trained (i.e. animals trained in low light were tested in high light levels and vice versa). Rats that experienced a decrease in light levels from training to testing were faster to sample ambiguous probes than animals that experienced the most negative shift in conditions, from low

to bright light. The experimenters concluded that this interpretive bias technique might be useful as an indicator of short-term changes in anxiety in rats and other non-human animals.

Not only do such tasks have the benefits of simpler behavioural responses to analyse and interpret compared with the un-conditioned tests such as the EPM and OF, but Burman and colleagues (2009) have shown that the bias can shift in response to conditions considered to affect anxiety-like behaviour, much like the relationship between anxiety and interpretive bias in humans. These tests may therefore have better translational validity than the common tests of anxiety-like behaviour, as interpretive bias can be assessed in both species, and potentially may use similar methodologies. Further work is therefore warranted to develop and assess different designs of this task (e.g. using intervals as cues – Matheson, Asher & Bateson. 2008), to test for age and sex differences in rats, and to see if the test has predictive validity when animals are treated with anxiolytic and anxiogenic medications. One problem that such research will have to address is how to train and assess adolescent rats over a relatively short period of time, given that the animal's ability to learn the task may well vary across the sub-stages of adolescence, as well as their performance in test trials (Spear, 2000).

2.2 Human Research

Not only is interpretive bias of interest to researchers in terms of comparing behaviour across species, but more work is warranted on its relationship to anxiety in humans. Given that interpretive biases are trainable in non-anxious participants and positive bias training can improve mood (e.g. Lothmann et al., 2001; Salemink & Wiers, 2011), interpretive biases could be an interesting target for therapy. To become a potential target for therapeutic techniques, more information must be gathered on how interpretive bias relates to anxiety, including how it may change or emerge during adolescence and any differences between the sexes. To do this, more tasks like Miers' and colleagues' AIBQ (2008) are required; tasks that have been specifically developed and tailored for use with adolescent populations, as opposed to adolescents' completing adult-targeted tasks. More specifically, changes in

interpretive bias need to be examined across the period of adolescence along with pubertal status, ideally in a longitudinal design, or at least by testing large samples of adolescents once during this period of life. As noted in the General Introduction and by Reardon and colleagues (2009), factors like chronological age must be accounted for in the analyses, preferably using the gold stand Tanner scale and clinical measures of anxiety. As puberty is a normative process rather than being pathological itself for anxiety, investigations into interpretive bias will help elucidate whether this negative cognitive bias has a role to play in mediating the anxiety-puberty relationship. Once the natural variations in interpretive bias and anxiety symptomatology among adolescents are elucidated, future work can then begin to consider interpretive bias as a target for therapy, and direct comparisons between the behaviour of adolescent humans and rats on interpretive bias tasks can be compared.

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Appendix 1

Publication arising from Chapter 3:

Lynn, D.A. & Brown, G.R. (2010). The ontogeny of anxiety-like behavior in rats from adolescence to adulthood. *Developmental Psychobiology*, ***52***, 731-739.